Light and Electron Microscopic Studies on Material obtained by Fine Needle Biopsy

A methodological study on aspirates from tumours of the head and neck region with special emphasis on salivary gland tumours

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INTRODUCTION

Material obtained by fine needle aspiration biopsy has been used in Sweden for many years in the diagnosis of tumours and has become a common tool in clinical practice. The pioneers in the development of this technique were Söderström (1952, 1966) and Franzén (1960, 1968), but many other authors (Zajiceck, 1965, 1970, 1974, Espositi, et.al. 1968) followed suit and numerous works on methodology and results have been published. All these studies are based on light microscopic observations. During the same period the development of transmission electron microscopy (TEM) prompted several pathologists to study sectioned material from tumours in the electron microscope, and this literature is rapidly increasing (Mori & Lennert 1969, Murad 1971, Mair & Tomé 1972, Ghadially 1975).

In 1973 Hagelqvist and Engström demonstrated that material obtained by fine needle biopsy could be used with good results for ultrastructural studies. In this preliminary paper diverse basic problems encountered in such studies were out-lined and some results presented. The very promising early results encouraged further study and the present publication is a direct continuation of the early paper. It is now based on both experimental and clinical material. The clinical material included in this book consists of tumours or other diseases of the head and neck region. It is the result of collaboration between the departments of cytology and otolaryngology. Important cooperation with the departments of oncology and of pathology made this study possible. In a parallel experiment a number of aspirates were collected from tumours not located within the head and neck region. This material is not included but provided additional information on the value of the method.

The preliminary report emphasized the importance of such factors as needle diameter, fixation- and embedding technique, etc.

The investigation also indicated that the time factor inherent in electron microscopy is of great significance. It is particularly important in cases of malignant tumours and certain viral and bacterial infections to find a technique which rapidly yields good results. These problems were subjected to analysis. Some of the questions studied can be summarized as follows:

Is it possible to find a fast and reliable technique for TEM-studies of material obtained by fine needle aspiration?

The preliminary publication showed that appropriate treatment of the material can give rapid results with the specimen quality still fairly good. An important question in this study is: **To what extent can the TEM-method provide information on cell-structure, -function, -activity, and on interrelations between tumour cells?**

It proved possible to make rather large semi-thin sections of material obtained by fine needle aspiration. This possibility is further studied and will be discussed. One question here is: **How representative is the material, and what quantity of cells can be found in each aspirate?**

Tumour material is often easily accessible to biopsy and TEM can then be conventionally used. Nevertheless in many cases it is not advisable to make a biopsy from a tumour, e.g. from a tumour in the salivary gland or when the aim is to follow the results of the treatment by irradiation or cytotoxic drugs. An important question then arises. **Will needle aspiration and TEM offer a possibility to follow the results of different kinds of treatment?**

It is established that the majority of the clinical material orginated from head and neck tumours. Particular attention is paid to a study of salivary gland tumours. It is of prime interest to determine **whether the present technique will add information which permits us to distinguish such tumours, and if this is the case, in what type of tumour can the method be of value.**

Our main problems are thus in abstract: How should specimens be obtained and handled to give both rapid and high quality results? Can study of such material add vital information concerning tumours and tumour cells surpassing the data provided by light microscopy?

Part I MATERIAL AND METHODS

This section was produced in cooperation with Berit Engström.

In this section we shall present a method for aspiration, fixation and embedding of material obtained by fine needle aspiration biopsy, which has been used in combination with conventional cytological smear techniques, often supplemented by material derived from biopsies or after operations. Particular attention was devoted to the construction of needles and to fixation and embedding. The technique was developed in animal experiments and on human material.

A. ANIMAL MATERIAL

a) **Qualitative tests:** We began our investigation with a pilot test, by sampling material from liver and salivary glands of guinea pigs in order to find a convenient vial in which the relatively small material could be processed. In addition, certain tests on osmolarity were performed. It could immediately be shown that aspirates from liver and salivary gland gave specimens in which the well known structure of these organs could be readily recognized. We found that some of our early tests produced good specimens, which stimulated us to continue our experiments with tumour material.

b) **Quantitative tests** with different biopsy needles: The test aspirates were made from 5 livers and 35 kidneys from pig — about 300 liver-aspirates and 1200 kidney-aspirates. These organs were chosen because the liver is known by experience to yield easily aspirated material, while it is more difficult to obtain tissue fragments from the kidney, because of its toughness.

Five types of needle were tested. (Fig 1D). The conventional, 0,7 mm Ø and 30 mm long injection needle (Mediplast) (A) used in everyday cytology in Uppsala. The second needle (B) is identical to the first except that the front opening was sharpened on one side on the assumption that the cutting front will increase the number of aspirated cells. The third needle (C) is a 0,7 mm Ø and 50 mm long subcutaneous needle (Mediplast). The fourth needle (D) is a 0,8 mm Ø and 40 mm long subcutaneous needle (Gilette) and the fifth needle a 0,8 mm Ø and 80 mm long injection needle (Mediplast).

Comparatively few punctures were made in liver (about 300), since it was established at an early stage that a sufficiency for embedding was obtained from this organ irrespective of the needle used for this easily punctured material. A total of 1,236 punctures were made in kidney, divided chiefly between needles A and B (709) as we were primarily interested in testing our ground needle and comparing it with the conventional A needle.

As we thought it possible, and also found by experience, that the needles could cause different degrees of bleeding during aspiration, all experiments which were measured and compared were made on freshly removed kidneys and livers from pigs. As far as possible each aspiration was made under identical conditions. The needle was passed back and forth seven times on a length of 10 mm, starting at the outer margin of the tissue. Then the pressure in the 20 cc syringe was equalized before the needle was retracted from the tissue. Then the tissue content of the needle was ejected into a small glass tube which had been weighed dry and empty. The aspirates were dried in a vacuum chamber with excess of drying agent present.

The dry weights of the aspirates were recorded and compared. The results from the needles B, C, D, and E were compared with that obtained by puncture with the (0,7 mm) A needle, which is used for daily routine punctures.

A histogram was plotted and mean values and standard deviations calculated (with the help of a computer). T-tests were performed.

B. HUMAN TUMOUR MATERIAL: GENERAL

The human material consists of 290 ''tumours'' some of which are inflammatory processes, mainly from the head and neck region. More than half of the ''tumours'' were localized to the salivary glands. The material was examined as follows:

a) **Conventional smears** of fine needle biopsies were stained with May-Grünewald-Giemsa for light microscopic cytological examination (Fig. 2).

b) **Aspirates for Epon-embedding** were taken (Fig. 1A), and ejected into small siliconized tubes (Fig. 1B). An Ellerman tube of the type used for Rh-determination was found to be appropriate for this purpose. At the time of ejection the needle was flushed with 2.5 % glutaraldehyde. The specimen was rapidly fixed in this way and often retained its form as a small core (Fig. 1C). Washing, post-fixation, dehydration and embedding were all done in the same Ellerman-tube.

c) **Biopsy:** In cases of later surgery a thin slice from the tumour was taken at operation and divided into small pieces of about 1 mm^3. These specimens were fixed in 2.5 % glutaraldehyde and embedded in Epon 812 (Luft 1961) in the same way as the fine needle aspirations.

C. HUMAN MATERIAL: TESTS FOR REPRESENTATIVITY

In an effort to analyse whether the aspirated material could be regarded as representative for the tumour examined a comparison was made with material obtained from ''surgery''-biopsies from the same case. For this investigation we chose tumours which were diagnosed from cytological smears as adenoid cystic cancer (ACC) or highly suspect ACC and studied them as follows.

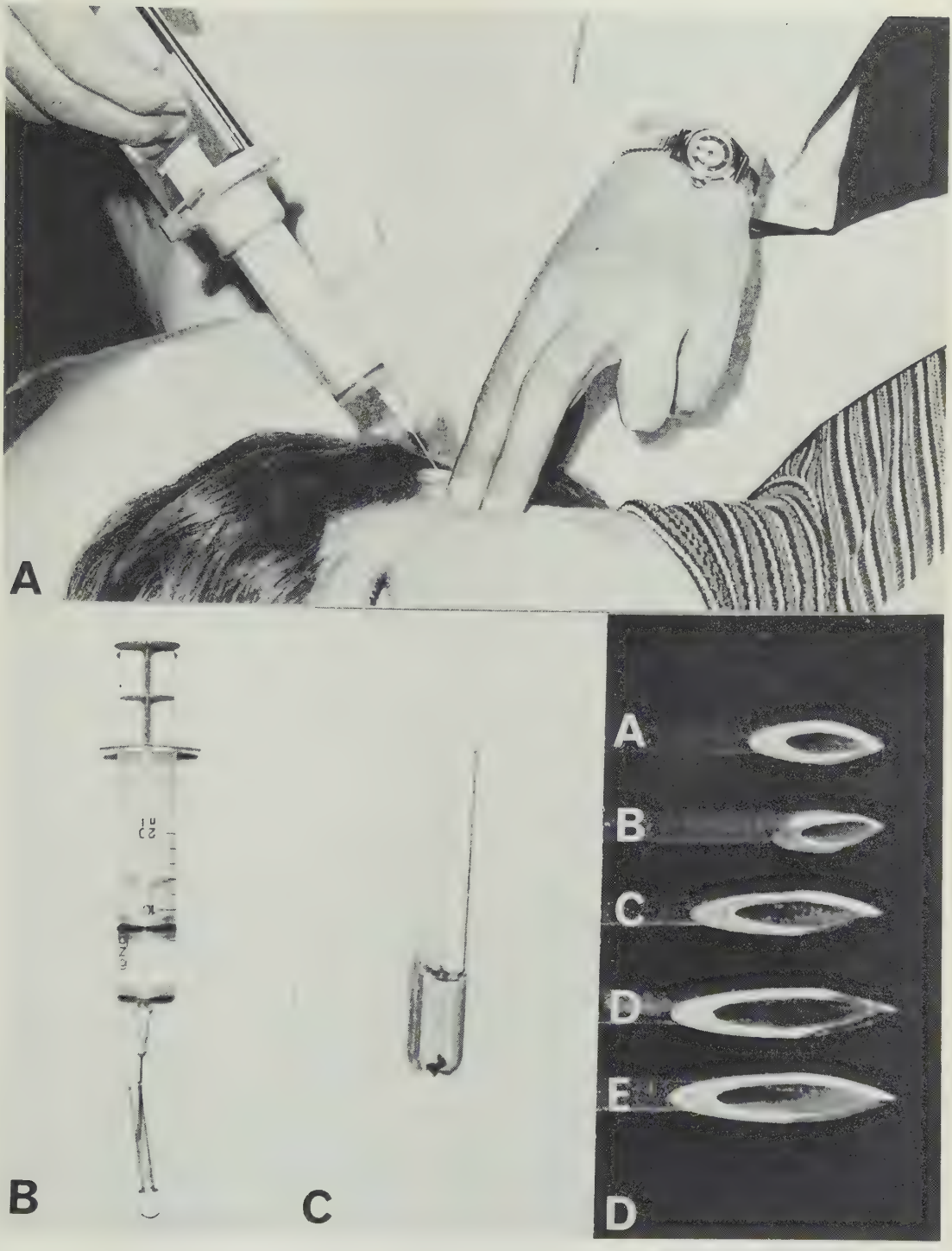


Fig. 1
A. Fine-needle aspiration with Frazén-syringe
B. Specimen being ejected into Ellerman-tube
C. Aspirate in fixation fluid (glutaraldehyde)
D. Different kinds of needle-opening

a) **Qualitative tests**

One fine needle biopsy was taken from each of three tumours, and these aspirates were fixed and Epon-embedded. Each was sectioned for TEM at five different levels — 15 different areas in all. Later, at surgery, a thin slice was cut from each of the three tumours. These slices were divided into small pieces. Five of these "surgery"-pieces were chosen at random from each of the tumours. Sections for TEM were cut from each of these small pieces — 15 areas in all. The areas sectioned were trimmed down to equal that of an aspirate specimen in area. About 12 micrographs at a magnification of 2000 X were required to cover every single TEM-section. The micrographs from aspirated and surgery material were mixed. Three experienced investigators tried independently to separate prints from aspirated material from those of surgery material.

b) **Quantitative tests**

In a effort to make a semiquantitative study of the cell material obtained by aspiration relative to the material derived from biopsy, seven cases of cytologically suspect or certain ACC were investigated. In sections chosen at random from both aspirate and biopsy 200 neighbouring cells were studied from each case. The cells were separated according to the criteria given below and histograms made. As will be further described in Part III, five cell groups were differentiated, mainly according to descriptions by Chisholm *et al.* (1975) and Hoshino and Yamamoto (1970). The following symbols were chosen: L-cells (light cells), L_1-cells (intermediate cells), D-cells (dark cells), D_1-cells (intermediate cells) and Sq cells (squamous epithelium cells).

D. FIXATION, EMBEDDING AND SECTIONING: GENERAL

a) **Standard technique**

In most cases we used 2.5% glutaraldehyde in 0.12 M phosphate buffer with pH 7.3 as fixative. In some cases we used cacodylate buffer but as its toxicity has been widely discussed, we preferred the phosphate buffer (Glauert 1975).

The fixation time varied from 1 to 4 hours (in room temperature). In a few cases the specimens were left in the glutaraldehyde for more than 48 hours, which did not seem to cause any major damage. After fixation the specimens were washed in the buffer or in isotonic saline, three changes during 1 hour. Postfixation was invariably carried out in 1.5% veronal-acetate buffered OsO_4, pH 7.3 (Palade 1952, Zetterqvist 1956, Glauert 1975) for 1 hour. The specimens were then washed three or four times during 30 minutes in isotonic saline. They were then dehydrated in ethanol of increasing concentrations (45—60 min) and finally brought into propylene oxide (2 × 15 min). In some cases, where time factors influenced the technicians work, we left the specimens overnight in 70% ethanol.The specimens were infiltrated in 1:1 mixture of propylene oxide and Epon 812 for at least 50 min. Then the lids were removed to allow the propylene oxide to evaporate before final embedding. Where a simultaneous embedding of several specimens was planned, the specimens first

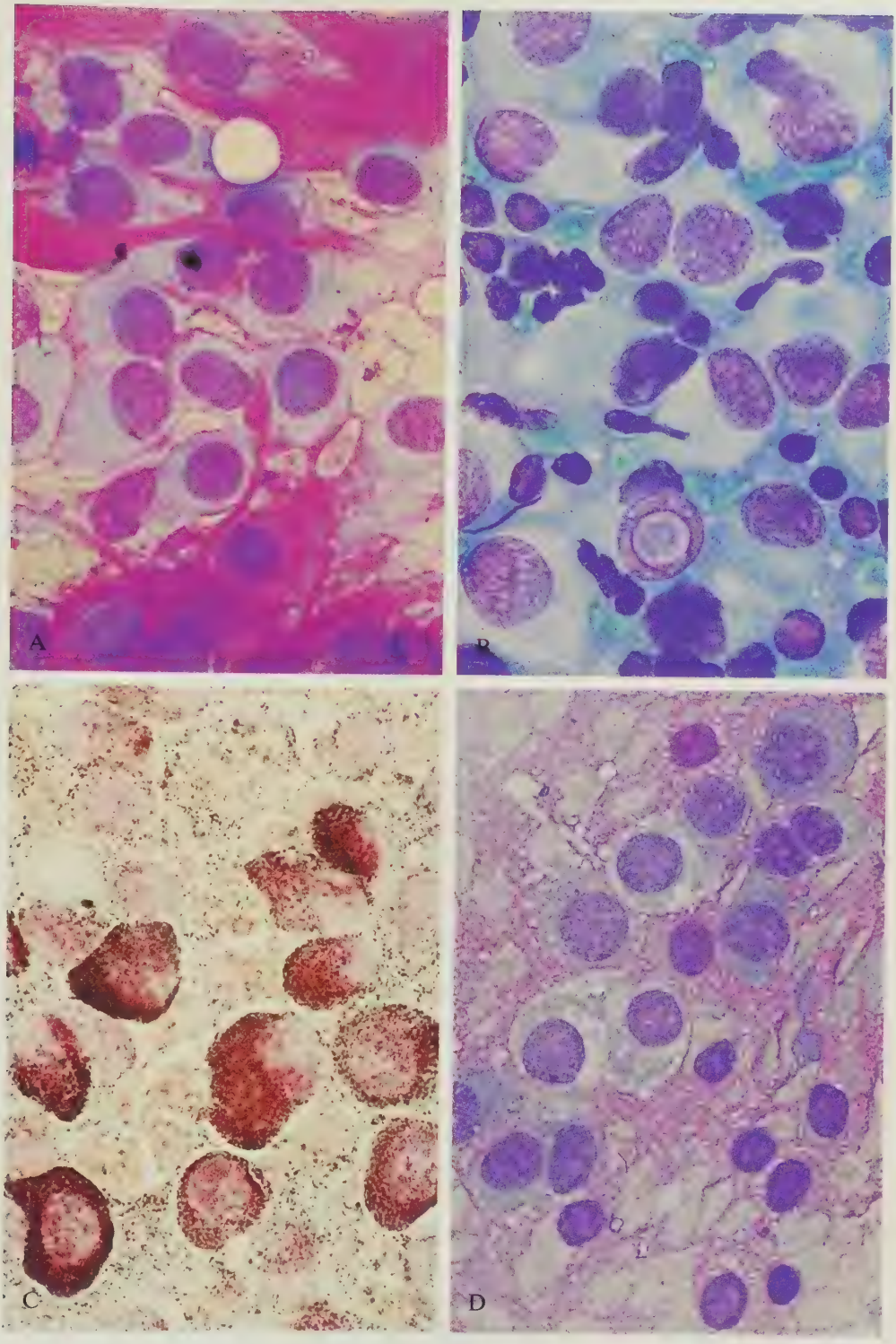


Fig. 2 Samples of some typical cytological smears.
A. from pleomorphic adenoma of the parotid
B. from poorly differentiated cancer from the parotid. Observe the pseudoinclusion in a nucleus.
C. Metastasis from malignant melanoma. Masson stain.
D. Cancer in the parotid gland, presumably of endocrine origin (compare Fig 25).

collected were left in propylene oxide — Epon-mixture for up to a few days. The final embedding was made in Epon 812 with polymerization for eight hours at 40°C, followed by 60°C for two days.

b) **Fixation and embedding for a "rapid" diagnosis**

When rapid processing was desired we used a technique for fixation and embedding modified after Johannesen (1973), Hayat & Guiaquinta (1970), Bencosme & Tsutsumi (1970). The specimens were fixed in 2.5% glutaraldehyde in 0.12 M phosphate buffer for 30 min at room temperature,followed by careful washing in isotonic saline. Postfixation was done in 1.5% OsO_4 in veronal-acetate buffer for 45 min. The specimens were dehydrated in increasing concentrations of ethanol for 40 min, followed by two changes of propylene oxide (2×5 min) and one change of propylene oxide and Epon in 1:1 concentration (20 min). The specimens were embedded in Epon 812 and polymerized at 70°C for 45 min, then in 105°C for 75 min. After polymerization the glass tube around the Epon often breaks spontaneously or will break if rapidly immersed in ice-water after which the glass is easily removed. When it remained intact, we cracked the tube in a vice with flat faces. The specimen can, with these times, be embedded within 5—6 hours. A somewhat slower method suitable for less urgent cases was also used. This is not as time-consuming and the preparation can stand polymerizing overnight, and be ready to section the next morning.

Table I illustrates the three methods used

Processing	"Rapid" 5—6 h	"Semi-rapid" 18—24 h	Standard 2—3 days
2.5% glutaraldehyde	30 min	1 h	1—4 h (48 h)
Buffer or isotonic saline	3×10 min	1 h	1—2 h
1.5% OsO_4	30—45 min	1 h	30 min—1 h
Buffer or isotonic saline	3×10 min	1 h	30 min—1 h
Dehydration:			
Ethanol 50%	2×5 min	2×5 min	5 min + 10 min
70%	2×5 min	2×5 min	5 min + 10 min
95%	2×5 min	2×5 min	5 min + 10 min
100%	2×5 min	2×5 min	5 min + 10 min
Propylene oxide	2×5 min	3 min + 15 min	2×15 min
Propylene oxide: Epon 812 1:1	20 min	30 min	50 min a few days
Polymerization	70°C 45 min followed by 105°C 75 min	70°C 45 min followed by 95°C 12 h or overnight	40°C 8 h followed by 60°C 2 days

All stages are carried out at room temperature.

Important: Since the material so often consists of small fragments the greatest caution must be observed when rinsing and changing different fixatives. It is advisable to use a Pasteur pipette with an external diameter of only 0.5 mm, which can be prepared by drawing a glass tube. This device prevents the small fragments from being sucked into the pipette and lost.

The cells and fragments were sometimes stirred up during one of the steps in the manipulation, and the specimen was then gently centrifuged.

c) **Sectioning**

The specimens were first studied under a preparation microscope. Sections (1—3μ thick) from regions of particular interest were made on an LKB ultrotome. These sections were mounted on glass slides and stained with 1 % paraphenylene diamine in water, or with 1 % solution of toluidine-blue in 1 % borax solution.

For electron microscopy (TEM) thin sections were made with a diamond knife. They were stained with 5 % uranyl acetate in redistilled water for 20 min at 55°C, and subsequently with Reynold's lead solution (Reynold 1963) for three min. A Siemens Elmiscop I A and a Philips 301 transmission electron microscope were used for this study.

E. RESULTS OF MATERIAL AND METHODS

The studies made on animals and human material clearly demonstrated that representative material of sufficient quantity and good quality could generally be obtained. In some cases the quantitative factor is of great interest, especially if it is difficult to obtain sufficient material. We therefore devoted considerable time to a study of needle design and aspiration technique.

The experiments with different types of needle (A, B, C, D and E) (Fig. 1 D), indicated that the shape of the needle is of interest, especially with regard to the grinding of the point, and the diameter and length of the needle.

When making aspirates from "difficult" material (kidney) we obtained different amounts of material with all types of needle. The different needles were compared, the conventional 0.7 mm injection needle being the basis of reference.

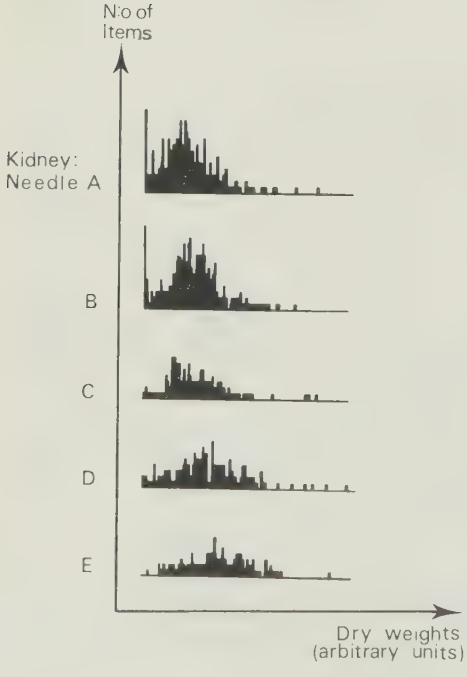
This reference needle, A, which we use for daily routine punctures to obtain smears for light microscopy, gave the poorest (least) result. Needle B, which is identical with A save that one side of the open tip has been ground to a cutting edge, gave 18 % more material. Needle C, which has the same diameter (0.7 mm) as A and B but has an opening ground lengthwise (subcutaneous needle), improved on A by 35 %. Needle D, which is ground like C, i.e. is a subcutaneous needle ground lengthwise but 0.8 mm in diameter, yielded 58 % more than A. Needle E is an injection needle, 0.8 mm in diameter and 80 mm long, ground at the opening like C and D. This gave 100 % more than A, i.e. twice as much yield (on average) per puncture. From "easily" aspirated material (liver) we obtained sufficient material for TEM,

irrespective of the type of needle used. Consequently only a few punctures were made with A and B on liver. If the yield from these two needles is assessed on easy and on difficult material then the needle gave five times as high a yield from the easy as from difficult material (kidney). See histogram (Table II).

The small core and fragments of tumour tissue obtained by aspiration are well suited for fixation, and very high quality is often found. With such small quantities of material it is important to exclude every possibility of contamination. We believe that *the use of a single tube* throughout the handling of the specimens constitutes a guarantee against contamination. This precaution also involves little risk of losing important material during processing.

With the method used it may happen that the glass tube does not break spontaneously, or on immersion in cold water after removal from the oven. The tube can then be broken by compression in a small vice.

There is a possibility that even better quality could be attained by changing the tube, but, as this could mean loss of material or contamination, we decided on the technique described, and use it daily with good results.



Kidney: Needle	n Sample size	$\bar{m}$ Sample mean	s Sample standard deviation
A	347	34.63	23.66
B	362	40.90	23.10
C	162	46.17	23.90
D	208	54.20	28.26
E	157	65.80	25.44
Liver: Needle			
A	50	205.60	93.18
B	50	149.36	59.45

Table II
Frequency distribution of dry weights of aspirates obtained by different needles.

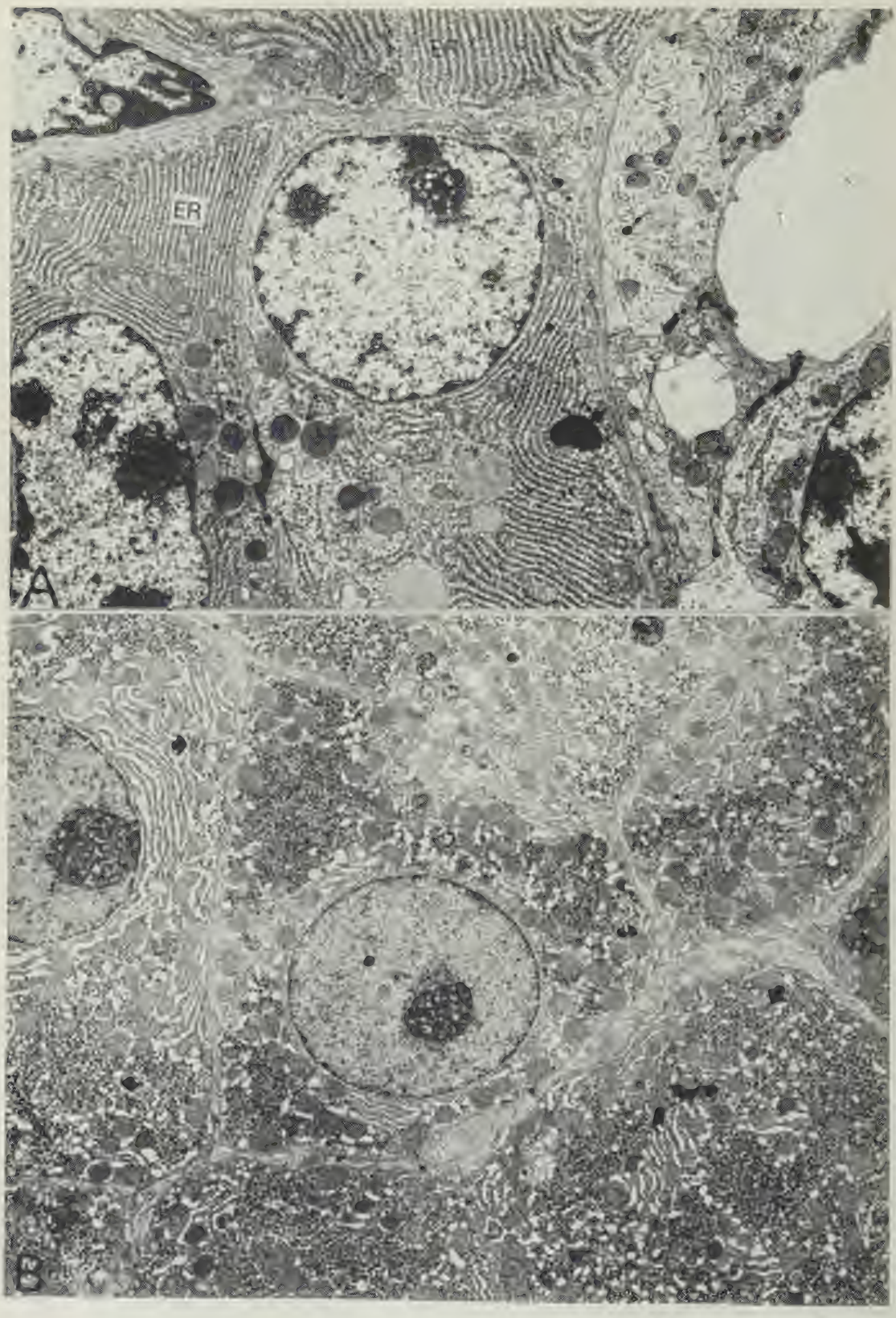
Osmolarity investigations.

The primary fixation consisted almost exclusively of of 2.5% glutaraldehyde in phosphate buffer, followed by secondary fixation with OsO_4 in veronal-acetate buffer. At the beginning of the investigation studies were made with regard to suitable osmolarity of the phosphate buffer; these were performed on guinea pig salivary glands (Fig. 3). Later in the course of the investigation two further series of tests were carried out on the same tissue, in which the molarity of the phosphate buffer varied between 0.06 and 0.19 M, with gradual changes of 0.01 M. On this material from normal salivary gland tissue the cristae and membrane systems of the mitochondria were, as a rule, best preserved at values between 0.12 and 0.14 M, corresponding to an osmolarity of 280—310 mOsm. It has long been known that the osmotic pressure differs in different cells, and likewise in different tumours (Palade 1956). In our tumour material we normally used a phosphate buffer which was 0.12 M, corresponding to an osmolarity of 280 mOsm. Naturally it was impossible to determine whether poor quality in membranes and mitochondria in a specific case depended on wrong osmolarity, since so many other factors in the embedding of the material influence the final quality.

By application of the **rapid embedding technique** the specimens could be made ready for electron microscopy within five hours of aspiration. The duration of fixation in glutaraldehyde could be reduced to 15 min, and the period in OsO_4 to 30 min, without major impairment of quality. The period of dehydration, however, must be maintained. We found no major structural differences in rapidly processed specimens when compared with material subjected to routine treatment (Fig. 4). We used the rapid process in more than 20 cases. The speedy processing does not seem to have any serious disadvantages if the specimens are small and the change from propylene oxide/Epon mixture to pure Epon carefully carried out to ensure that no propylene oxide remains. Thus the technique can be used for swift diagnosis when the time factor is crucial. In cases where the timing is less important, however, it is necessary to develop techniques where the methodology is controlled by the dictates of quality.

Semi-thin sections

In the preparation of sections for TEM it is usual to make semi-thin sections first, in order to locate special regions for ultra-thin sectioning. This is necessary when representative regions are desired or the material is small and perhaps mixed with blood. The material obtained by fine needle biopsy may, as stated, vary considerably in amount, but specimens frequently contain much more material than was expected. In such cases, when the material is sufficient, a study of semi-thin sections (1—3μ) may be of value. It is common to find large numbers of cells, well fixed and interrelated with neighbouring cells. The cells are often arranged in large clusters, sometimes retaining the cyto-architecture of the tumour. An example is illustrated in Figs 5 and 6 A. As a single semi-thin section may contain several hundreds to thousands of cells, it offers an opportunity to observe many details, such as form and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A
B

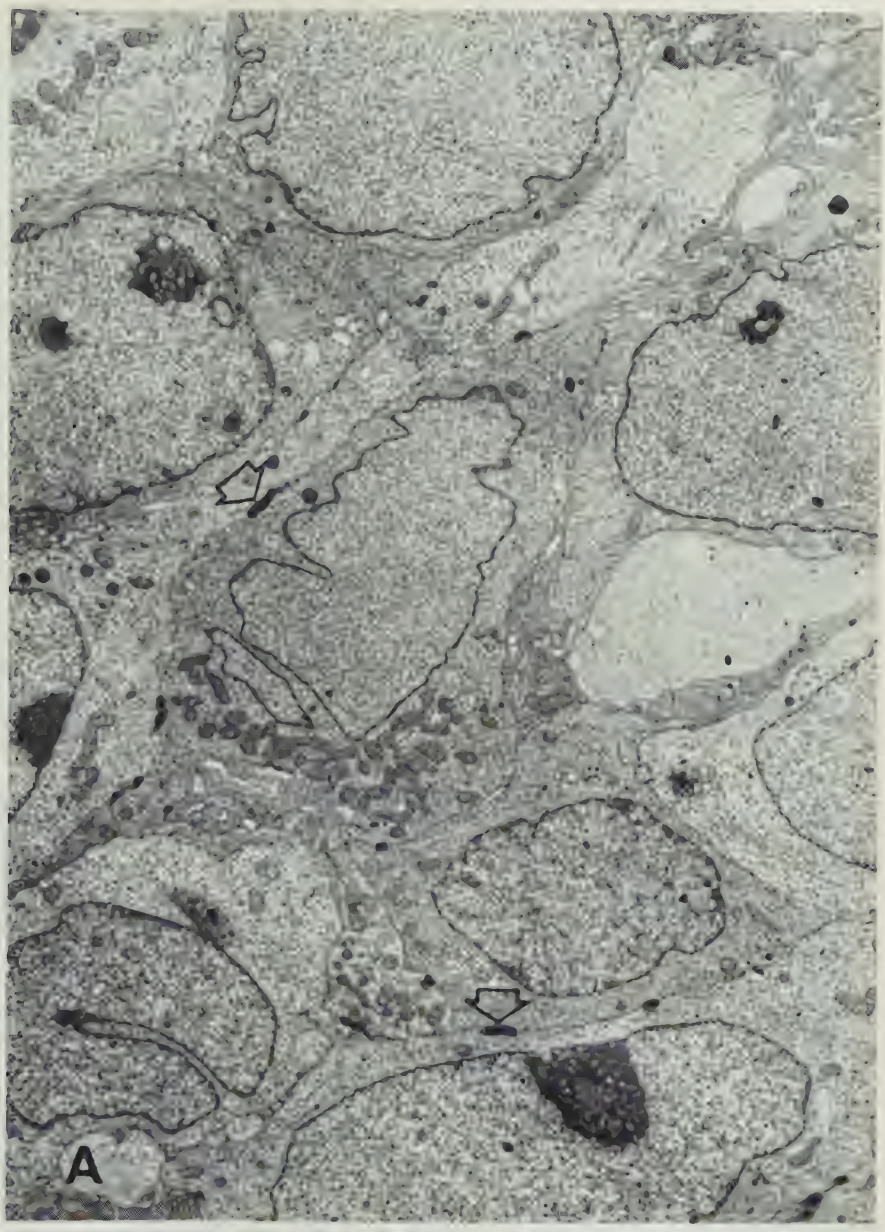


Fig. 4 Micrograph from a case of malignant "lympho-epithelial lesion" where desmosomes (open arrows) are found between the cells.
This specimen is handled according to the rapid embedding technique. Micrographs of this quality can be made within 5—6 hours after aspiration. (6 500 ×).

Fig. 3 Two micrographs from our pilot studies. A, Normal salivary gland, from guinea pig (6 500 ×). B, Liver puncture from a patient with symptoms of rejection of his transplanted kidney (4 500 ×). These two micrographs are to demonstrate that the general texture is retained in the aspirate.

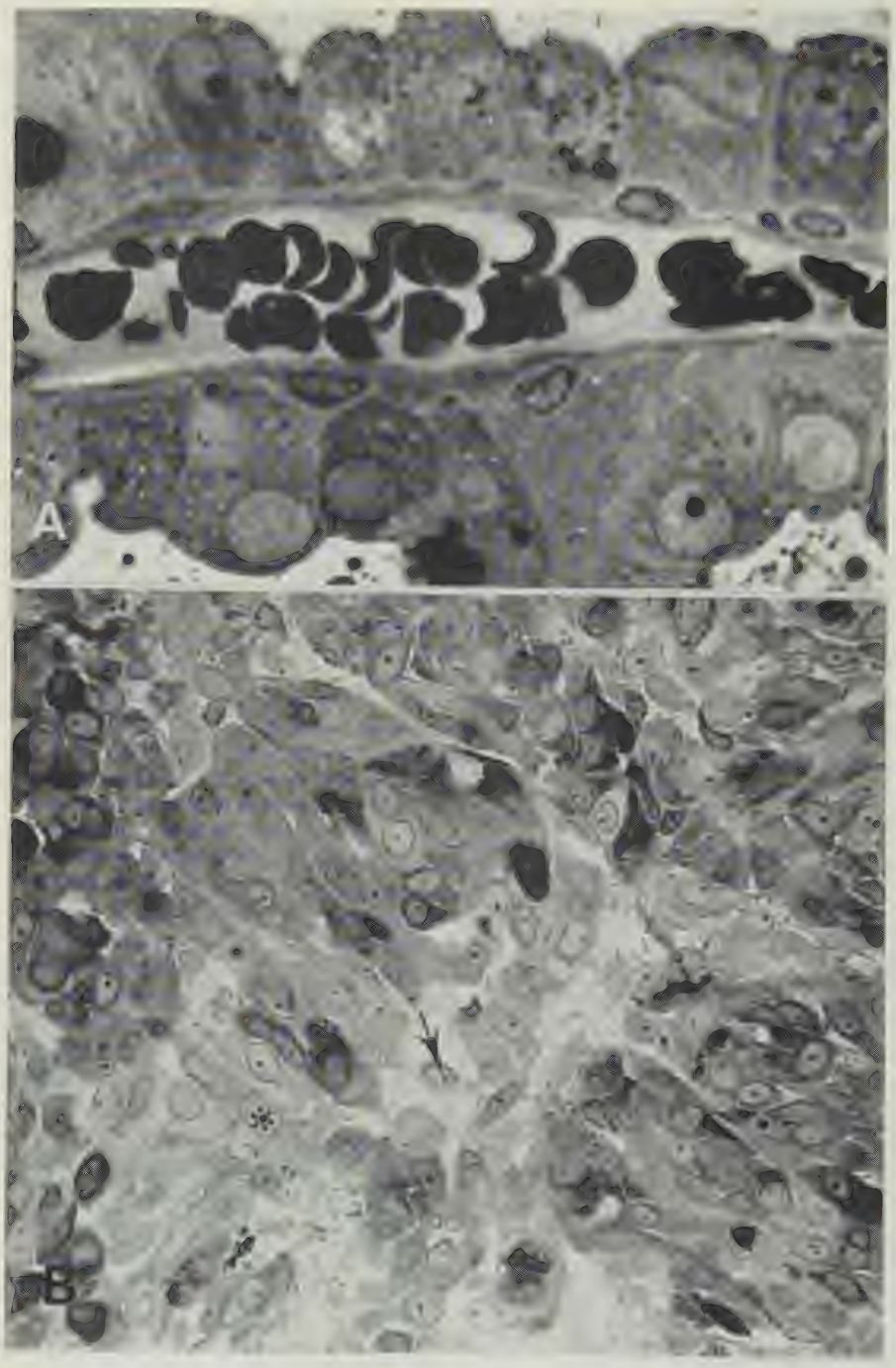


Fig. 5 Epon embedded tumour material, semi-thin sections. A. Adeno-papillary cancer of the parotid gland (1550×). B. Unclassified cancer of the parotid gland (200×). Phase-contrast microscopy. These micrographs demonstrate that it is possible to get good information on a tumour and its cells. Many details of the cell cytoplasm, nuclei (arrow) and nucleoli (*) can be recognized. Certain information concerning the cellular arrangement is also obtained.

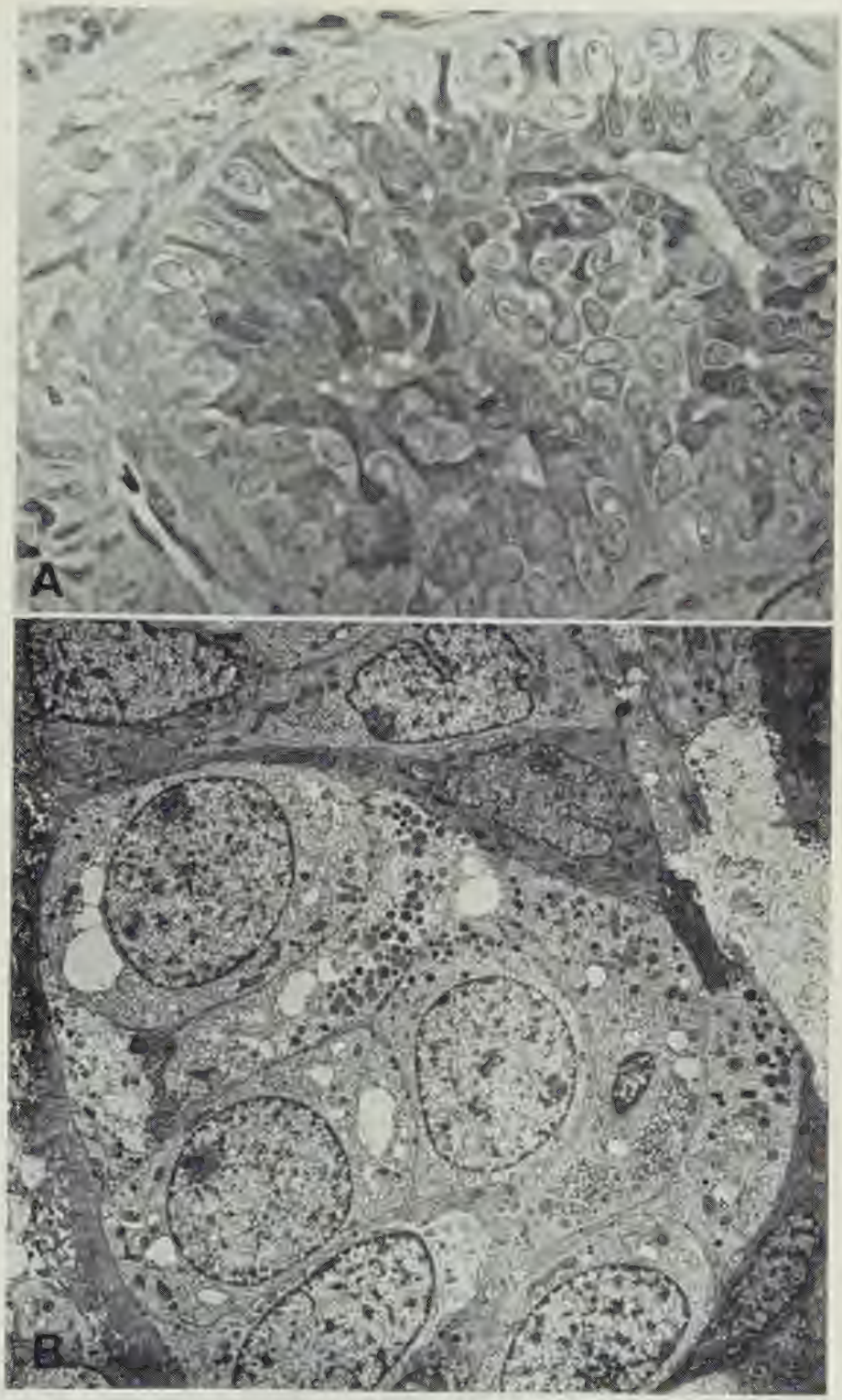


Fig. 6 Micrographs from a case of sialosis in the parotid gland. A is a semi-thin section and B a TEM-micrograph. There is good agreement between light and electron microscopy. (A 710×; B 4 100×).

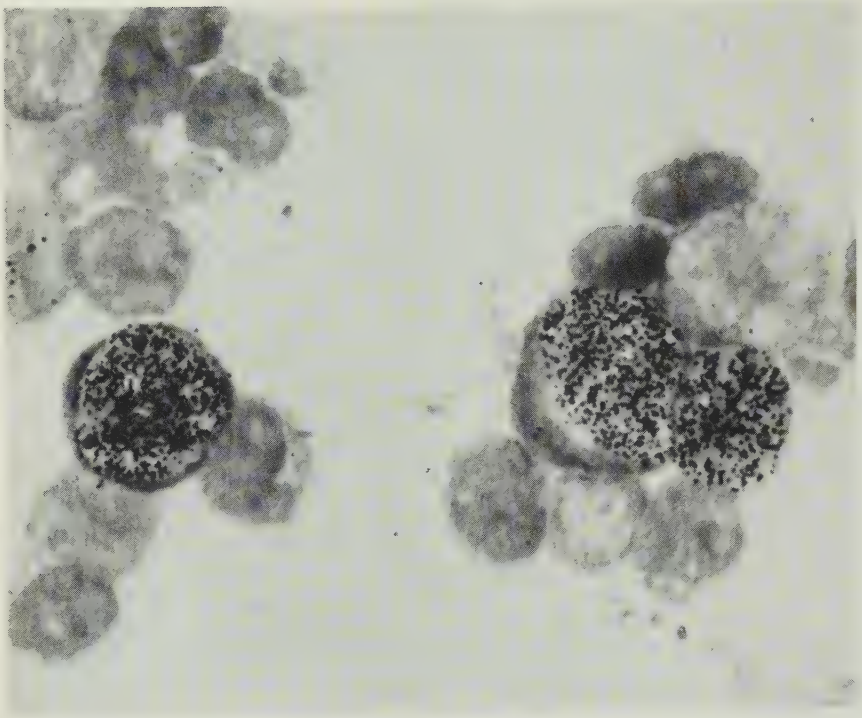


Fig. 7 Autoradiography of tritiated thymidine in a case of malignant lymphoma. The uptake of isotope is visualized by the dark grains in the cells. Aspirate and light microscopy.

size of the cells and their nuclei, cytoplasmic granules or inclusions, relation to vessels or microcysts. When we began our studies we used these sections for TEM localization only.

We found, however, that they can be of interest for the evaluation of the tumour as well. A typical example is seen in Fig. 31 B. showing a semi-thin section from an adenoid cystic cancer which had been irradiated. Material from the operation showed no tumour cells present, while our semi-thin section clearly reveals the presence of tumour cells and the characteristic pseudocysts with their basement membrane-like wall.

During our studies of the aspirates, and also when studying materials from operations we had access to various methods not described here. These include autoradiography with tritiated thymidine and scanning electron microscopy (SEM). We have not included this part of our material, but Fig. 13 B illustrates a SEM-micrograph. It should be mentioned that for special purposes, such as mycosis or the study of surface structures, the SEM-technique may be an important adjunct to other methods here described. Autoradiography with tritiated thymidine was used in the study of the results of irradiation treatment, but the evaluation of this material involved elements of uncertainty and our ivestigations must therefore be continued. Nevertheless we were able to demonstrate that aspirated material can be used for at least light microscopic studies of this type. This technique will aslo be studied further.

Part II. TRANSMISSION ELECTRON MICROSCOPIC STUDIES ON HUMAN MATERIAL

A. CELL STRUCTURE. GENERAL.

Clinical diagnosis of tumours was for many years dependent mainly on light microscopic observations, but during the last decade more and more information on tumours and tumour cells has been derived from electron microscopy. Transmission electron microscopy was the chief technique used in these studies. The combined use of light and electron microscopy has enriched our knowledge. In the present study we confirmed Ghadially's finding concerning tumour cells: "It is amazing how much more one can discern with the light microscope once their ultrastructural features are recognized". The cytological study of smears from tumours is to a great extent based upon the form and structure of cell nucleus and cytoplasm, and the interrelationship of tumour and host cells. The same factors are of great interest also in TEM studies, but the possibility of discerning the detailed inner structure of the cell nucleus and cytoplasmic organization adds a new dimension.

Diverse important factors appear in the literature with regard to TEM studies of material from surgery specimens, but also in the present investigation, factors which are of diagnostic interest which may become more so as our knowledge of their importance increases.

A striking feature is the detailed information on cell borders (Fig. 8) and cellular relationships which TEM can offer. TEM provides an excellent opportunity of observing cell junctions and interdigitations (Fig. 9), while cytoplasmic organelles such as microvilli, inclusions etc. can be radily discerned on free cell surfaces.

Many authors have reported a reduced number of junctions between some types of tumour cells and wider intercellular spaces in some tumours have also been observed. On the other hand, a positive finding of many desmosomes in a tumour of uncertain etiology may be of importance in the differential diagnosis between a carcinoma and a sarcoma. In Fig. 4 presence of desmosomes in a case of poorly differentiated salivary gland tumour excluded a malignant lymphoma, and contributed to the final diagnosis of malignant lymphoepithelial lesion. We encountered a few cases with this diagnosis, and they are now under further study. TEM examination of the cell surface of tumours may show special cytoplasmic features, such as cilia and microvilli, as indications of differentiated surface activity (Fig. 29 B). A loss of such specialized cell function may be a sign of malignancy. Similarly an increased incidence of atypical microvilli, seen e.g. in "hairy leukemias" (Bessis 1977), may be of significance for the diagnosis.

Fig. 8 Myeloma cell, from the bone marrow, fine-needle aspirate. This micrograph shows how TEM permits a study of the structure of cell nucleus and cytoplasm. Mitochondria (*M*), endoplasmic reticulum (*Er*) and cytoplasmic granules (*G*) as well as cytoplasmic protrusions (*P*) from the cell surface are evident. (14400×).

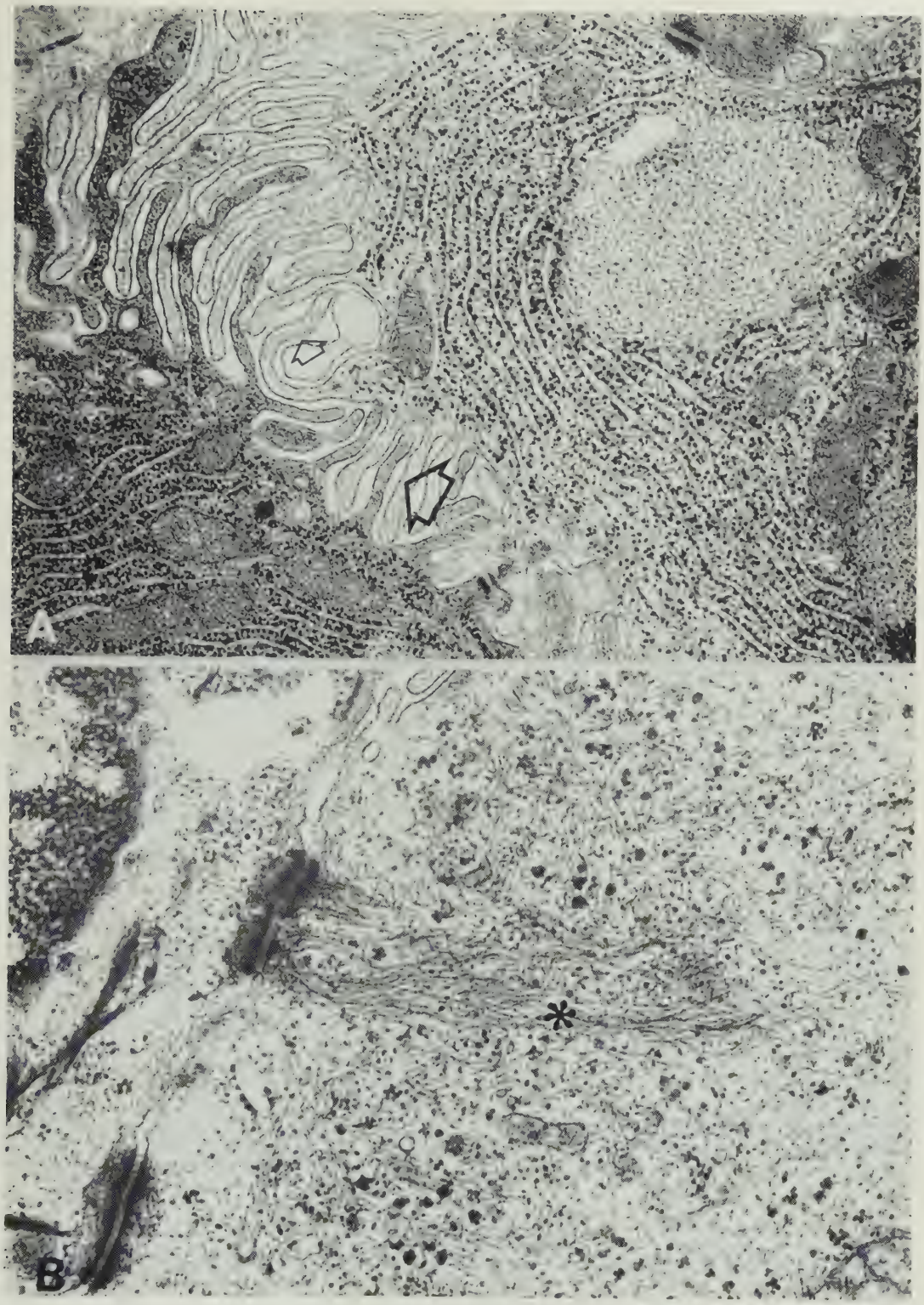


Fig. 9
A. TEM-micrograph showing interdigitation (arrow) between two salivary gland cells, (31 000 ×).
B. Desmosomes with long tonofibrils(*) seen in a case of pleomorphic adenoma, (45 500 ×).

B. SPECIFIC CELL STRUCTURES AND VIRUSES

The cell surface is not the only subject of interest, since the form and structure of the cell nucleus emerges more distinctly in TEM than in light microscopy. It is astonishing how often light microscopy implies that the nucleus of a tumour cell is a regular sphere, whereas TEM proves that many are irregular and indented, and perhaps contain pseudo-inclusions. Three nuclei and various pseudo-inclusions are illustrated in Fig. 10. The full significance of the structure of the complicated infoldings in Fig. 10 A or the protrusion in 10 B cannot yet be understood. It is assumed to correspond to wider area of contact between the nucleus and the surrounding cytoplasm of importance for nucleocytoplasmic exchange, or is perhaps an indication of enhanced metabolic activity.

Pseudo-inclusions in the nuclei are comparatively common in malignant tumour cells, and were observed in myelomas (Fig. 10 B and C), in malignant melanomas, and in papillary carcinomas of the thyroid. These pseudo-inclusions can be clearly discerned even in ordinary cytological smears studied under the light microscope, (Fig. 2 B) but only when using TEM is it possible to distinguish the nature of the structures contained within them. A high frequency of pseudo-inclusions in naevi and malignant melanomas was descibed by Sobel et al. (1969), who found them in 8 of 10 malignant melanomas, and 27 of 34 naevi.

The chromatin of the nucleus in our specimens varied widely in different kinds of tumour and also in different parts of the same tumour. Presumably increased knowledge of chromatin and nucleolus structure will improve our possibility of evaluating the potential growth of tumour cells.

In the studies one of the prominent features revealed by TEM was the form of the nucleolus in individual tumours. Enormous nucleoli could be seen in a case of maxillary cancer (Fig. 11). In other cases we observed a rich variety of nucleolar structures in different types of tumour, and even in tumours where the clinical diagnosis was the same. Different types of nucleoli are illustrated in Fig. 12. Figure 12 A shows an enlarged, centrally situated nucleolus, in contrast to that in Fig. 12 C, which displays "nucleolar margination" in a tumour cell from a malignant lympho-epithelial lesion in the parotid gland. The latter form was regarded by Ghadially (1958, 1971, 1975) *et al.* as a sign of rapid cell growth and increased protein synthesis. Nucleoli of the type illustrated in Fig. 12 B were observed by Luse (1961) in certain brain tumours.

A less common intranuclear inclusion is shown in Fig. 13. This "concentric laminated inclusion" was found in an aspirate from a submaxillary gland infected by actinomyces Israeli. The importance of such inclusions, seen by Tandler and Denning (1969) in mucus producing cells in human salivary glands, and by Erlandsson and Tandler (1972) in adenocarcinomas of the parotid gland, is not known.

We observed another spiral-like inclusion in a case of malignant lympho- epithelial lesion. Here too the significance is obscure. Similar inclusions were seen by Zelickson and Lynch (1961) in a case of kerato-acanthoma, and classified as caused by a virus. Similar nuclear bodies were described by Bouteille et al. (1967) as evidence of cellular hyperactivity of multiple origin.

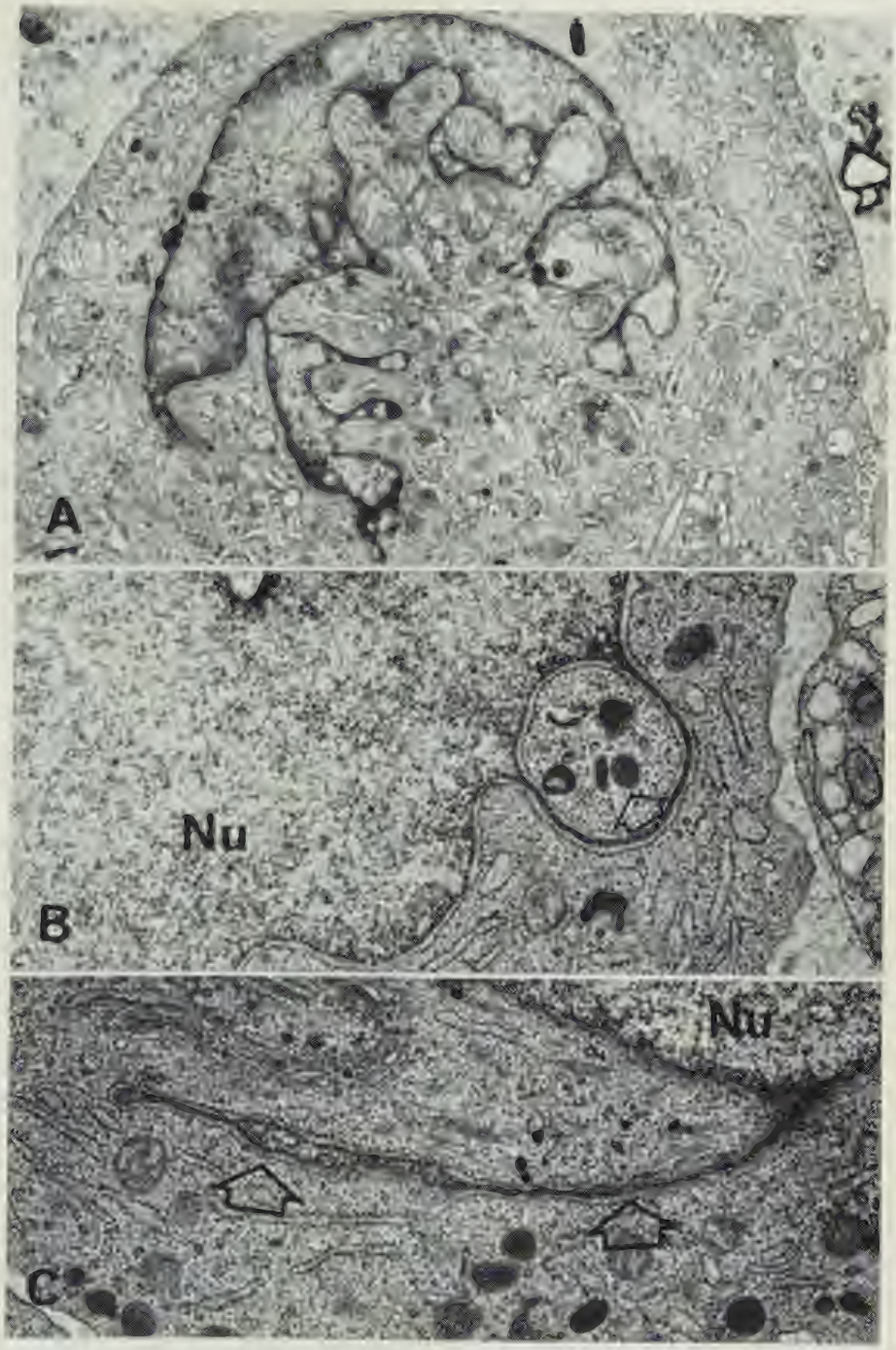


Fig. 10 Three micrographs demonstrating the structure of the nucleus with TEM on fine-needle aspirates. A is a very irregular nucleus in a case of poorly differentiated salivary gland cancer (12900×).
B is a pseudo-inclusion (arrow) into the nucleus (*Nu*) of a myeloma cell (2400×) and C is the irregular nuclear envelope (arrow) in myeloma cell from the same case as B. (2400×).

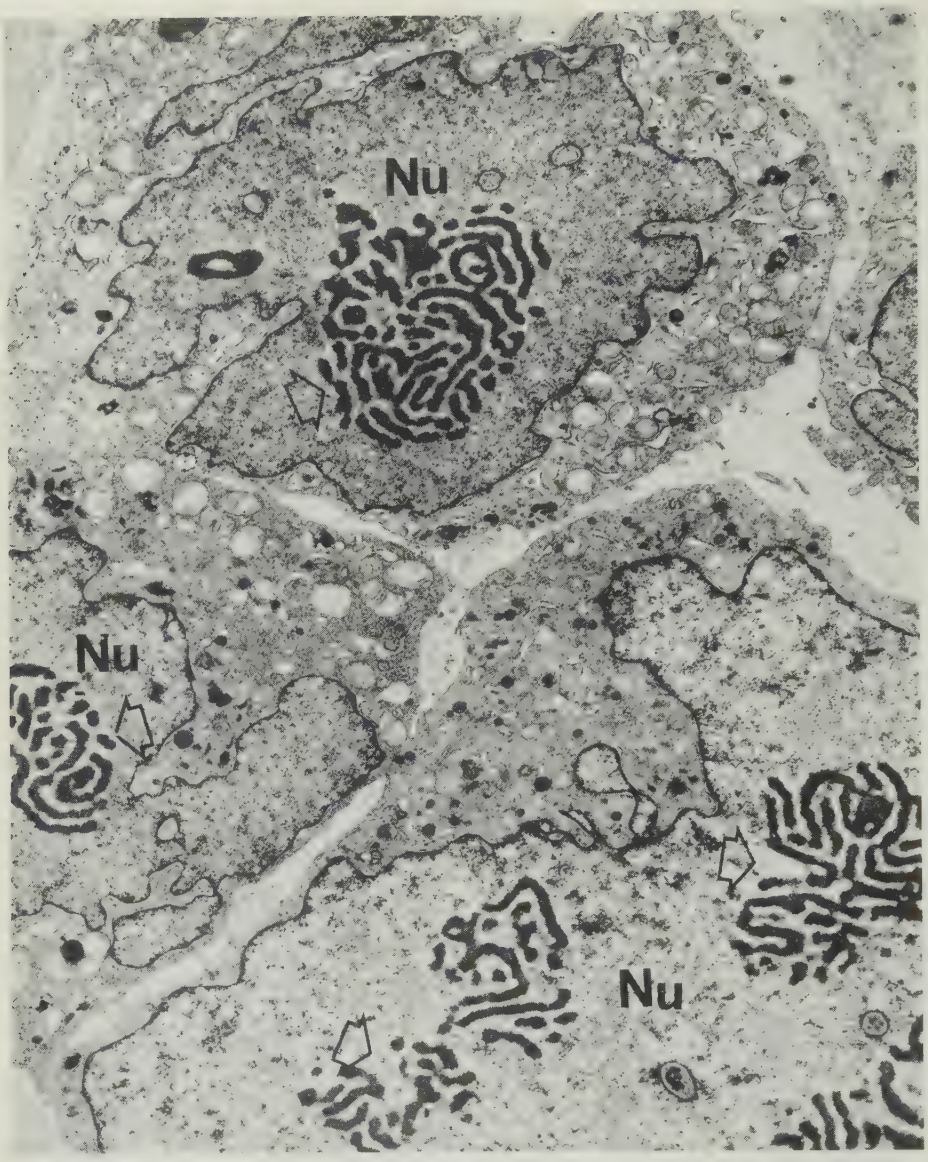
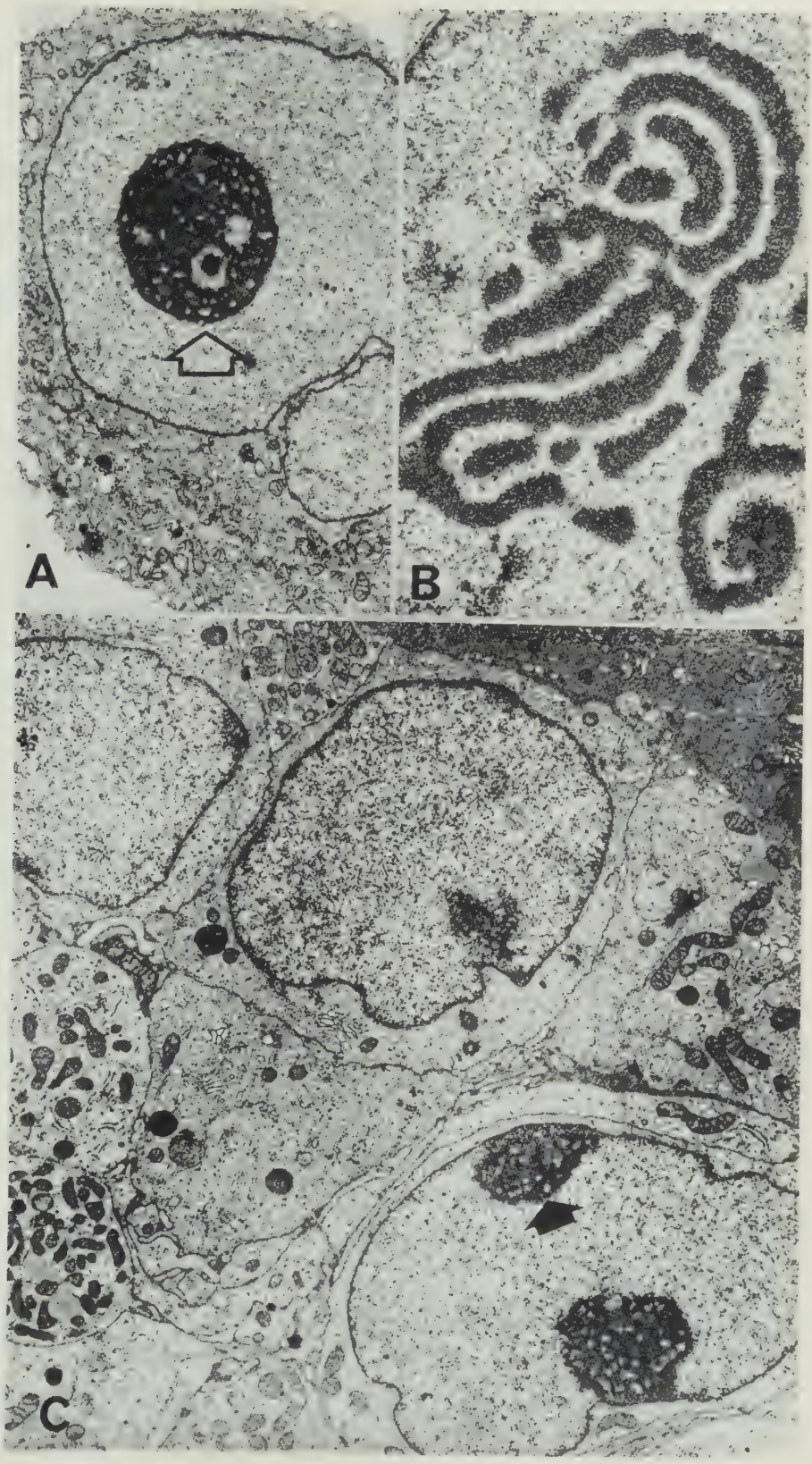


Fig. 11 TEM on an aspirate of a rapidly developing maxillary cancer with large nucleus (*Nu*) and enormous nucleolar structures (arrow). This case showed many mitotic divisions and the micrograph indicates high mitotic activity. (7100×).

Fig. 12 Three micrographs present different forms of nucleoli: A is from a case of metastatic sarcoma, (7000×), B from a maxillary cancer, (24000×) and C from a malignant lymphoepithelial lesion, (10400×). Arrows in A and C point at the nucleoli.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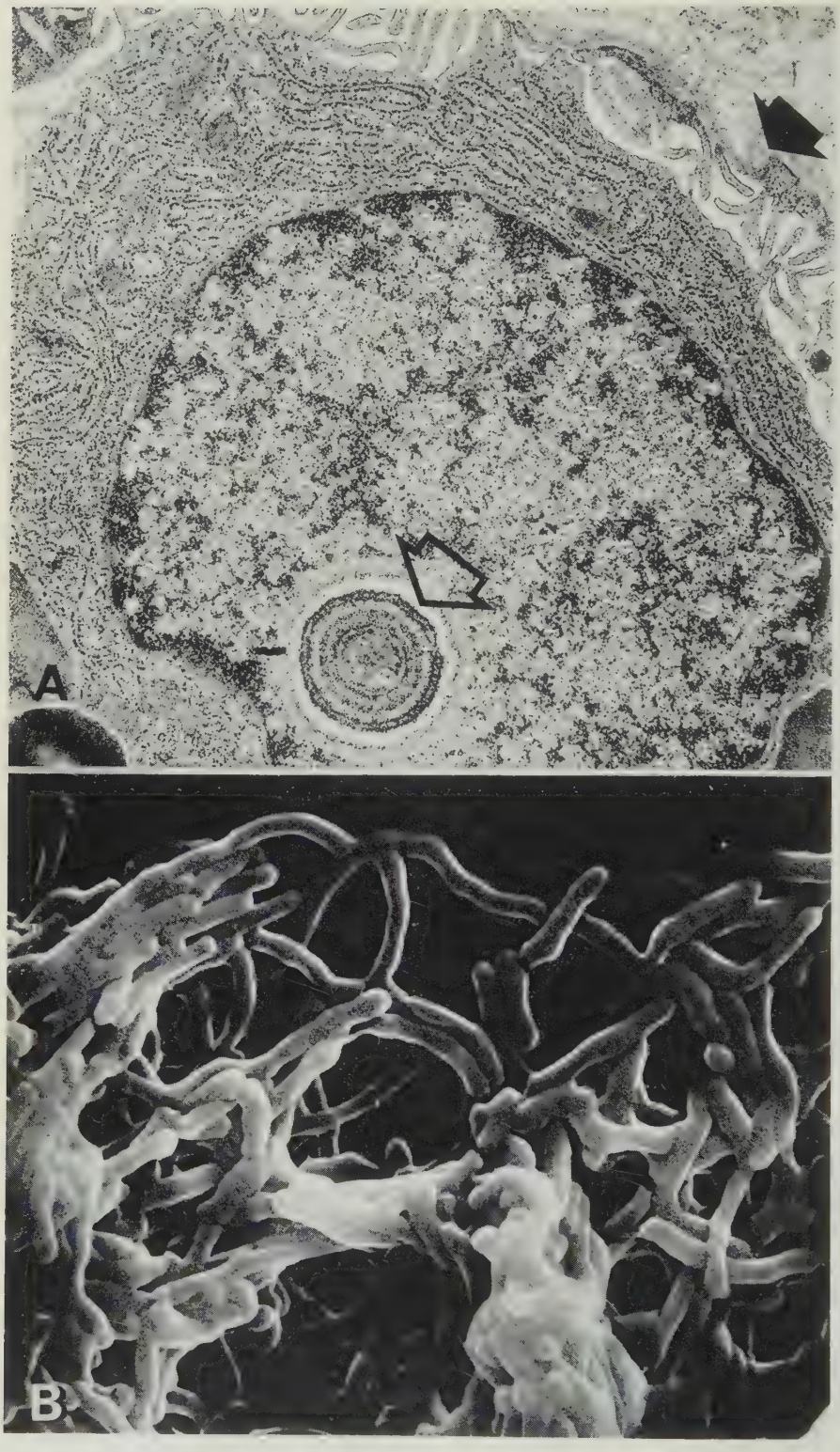


Fig. 13 A. TEM-micrograph and B. Scanning electron micrograph from a case of actinomycosis (*Actinomyces Israeli*). In A the open arrow indicates an intranuclear concentric inclusion and closed arrow cell junctions, (18 400 ×); in B the mycelium can be seen. (8 500 ×).

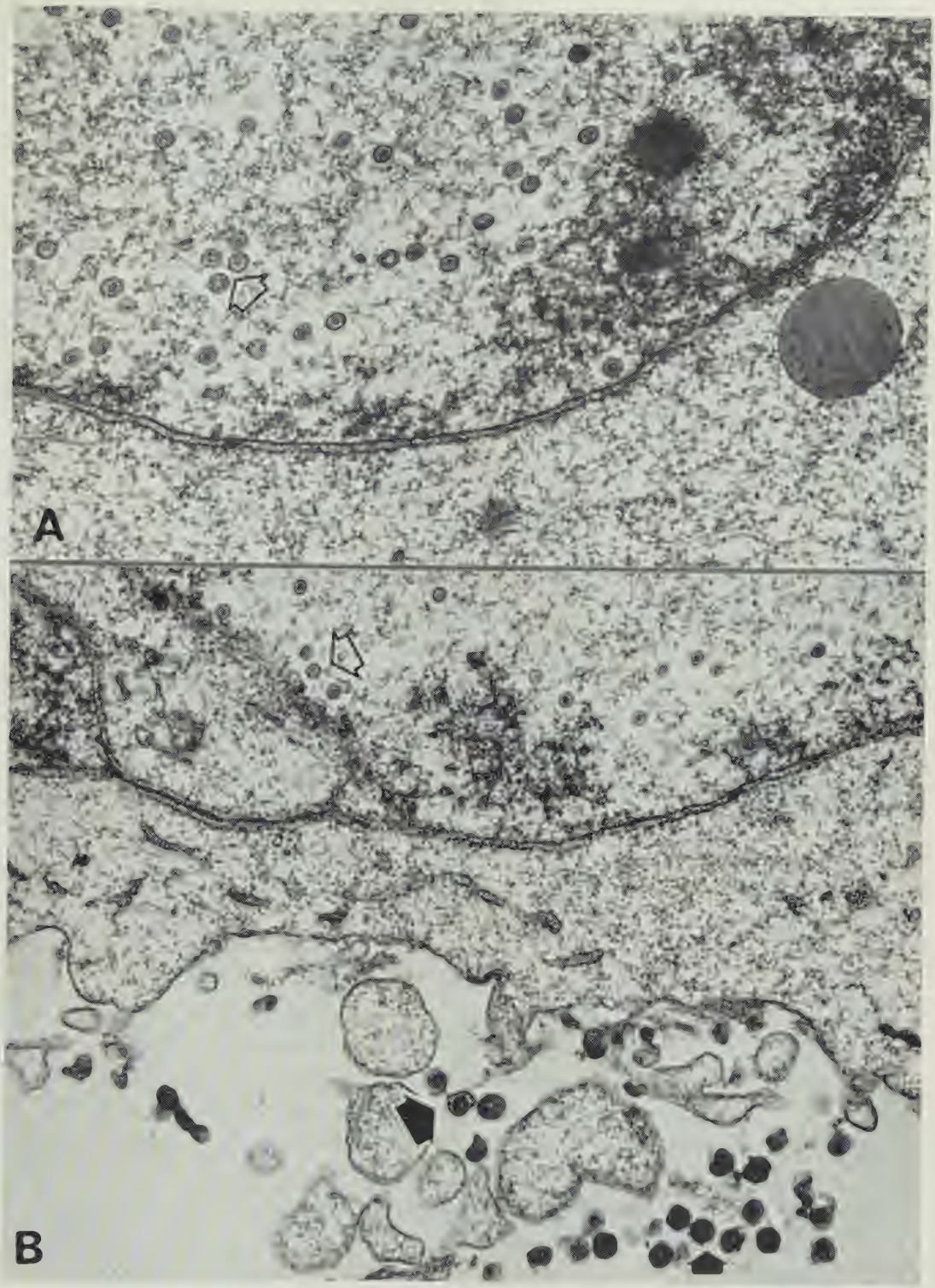


Fig. 14 Two TEM-micrographs showing viruses in a case of herpes encephalitis. Fine-needle aspirate and TEM was carried out in this case which was supposed to be a brain tumour. With the rapid method we could within 6 hours demonstrate virus in TEM. In A there are viruses in the nucleus and in B are seen viruses both in the nucleus and extracellulary. (A. 36 400×, B 28 000×).

Fig. 15 Vesiculated endoplasmic reticulum (*Er*) surrounding a Russel body-like stucture in a case of myeloma. It is probable that the reticulum is distended by newly formed pathological protein, (24000×).

Viruses represent a special nuclear or cytoplasmic inclusion which can be directly recognized by TEM. An example from our material is illustrated in Fig. 14, where fine needle aspiration performed at operation of a case of suspected brain tumour, followed by TEM, showed viruses. In this case the rapid embedding method was used, and TEM examination took place within six hours after aspiration. The virus was of the *herpes simplex* type. The micrograph shows that inside the nucleus the central core of the virus is delimited by a so-called capsid. To the viruses which have left the nucleus one more layer has been added by the nuclear membrane.

The cytoplasm of cells in the aspirates may vary in quality of fixation but this is generally good, so that cytoplasmic organelles can be studied in detail. The rough endoplasmic reticulum (Er) is clearly defined in Figs. 8 and 15. In this case the plasma cell from a myeloma shows many other details of cytoplasmic differentiation.

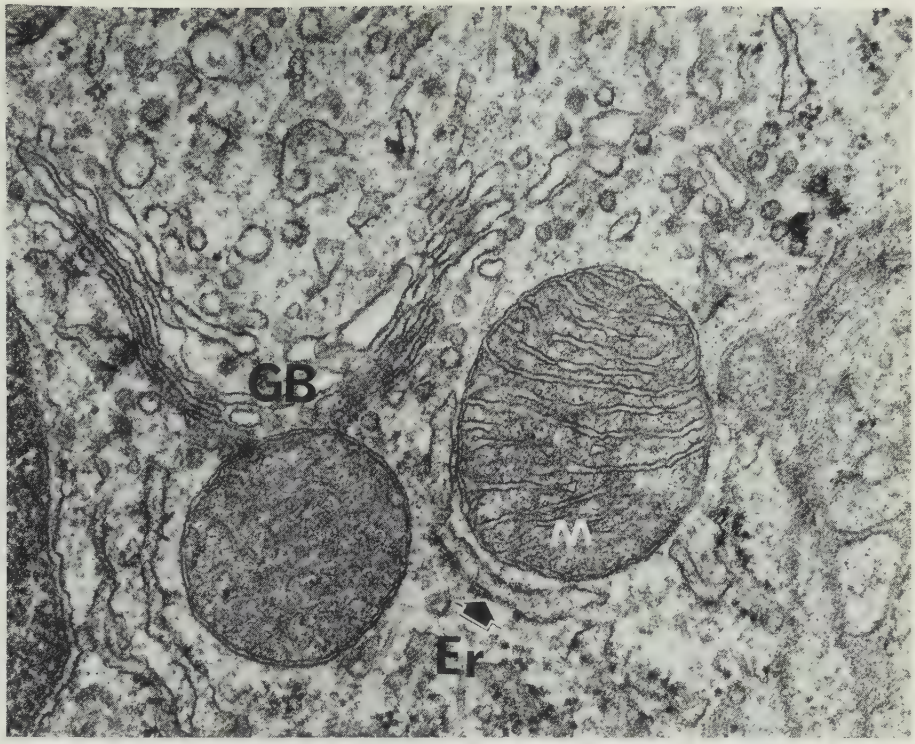


Fig. 16 Mitochondria (*M*), rough endoplasmic reticulum (*Er*), and a Golgi complex (*GB*) from a case of mucoid adeno-papillary cancer in a salivary gland. (46000×).

The presence of a well developed endoplasmic reticulum is regarded as evidence of advanced RNA synthesis. A rich rough endoplasmic reticulum is a normal finding in many secretory cells, as in salivary glands (Fig. 3 A and 9 A). The reiculum may vary considerably in form and extent in tumour cells, which is presumably directly related to protein synthesis.

Another tumour with pronounced secretory capacity and consequently a well developed endoplasmic reticulum is seen in Fig. 16, which shows a tumour from a mucous adenopapillary salivary gland cancer. There is also a well developed Golgi complex and secretory vacuoles signifying high secretory production.

Another arrangement is demonstrated in Fig. 17 B where the endoplasmic reticulum encloses the mitochondria, probably to allow of a more rapid exchange of energy. A rich endoplasmic reticulum is shown in Fig. 17 C, which is a TEM microphotograph derived from an aspirate from a lymphatic gland metastasis in the neck from an endometroid cancer. Light microscopy of the cytological smear revealed an abundance of "lymphoglandular bodies" (Söderström 1966)

Yet another type of "lamellar bodies" almost resembling smooth endoplasmic reticulum derived from a liver tumour is illustrated in Fig. 17 A.

Fig. 17 Three different forms of endoplasmic reticulum. A, An aspirate from a hepatoma, B, A mucous adenopapillary carcinoma in the parotid gland and C, a lymph node metastasis of an endometroid cancer. In A there is a smooth reticulum, in B a rough endoplasmic reticulum arranged around mitochondria (*M*), and in C a very richly developed rough endoplasmic reticulum, indicating a rich protein synthesis. (A 16000×, B 28000×, C 28000×).

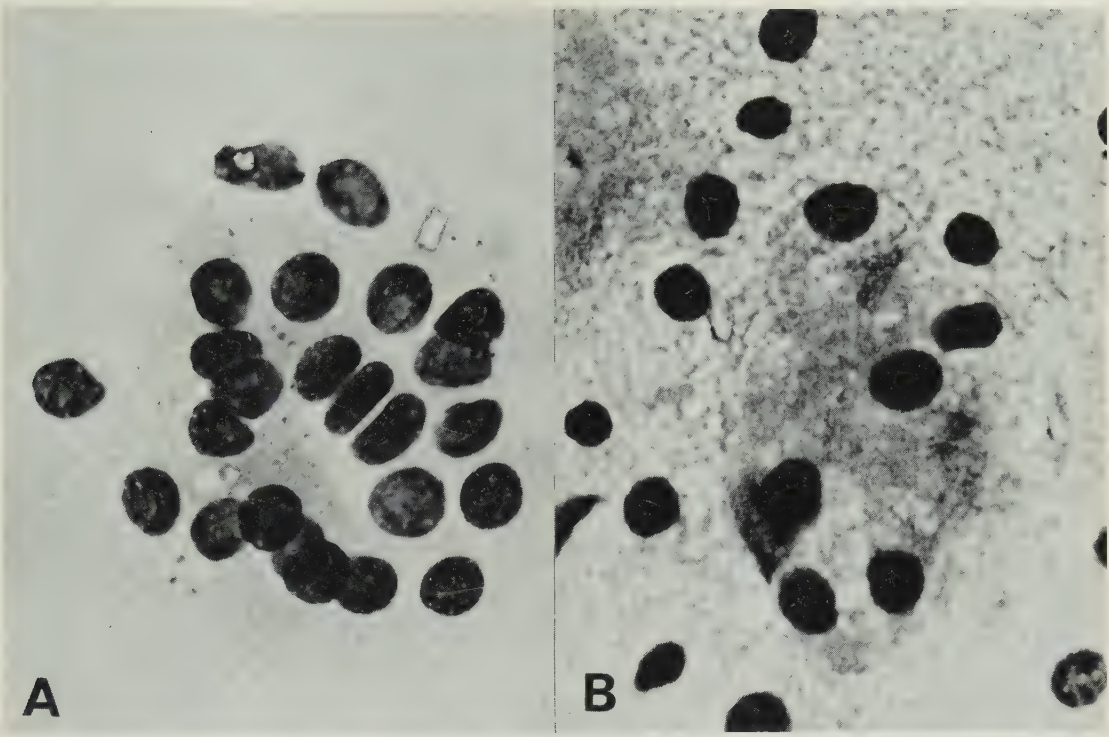


Fig. 18 Light microscopic pictures from smears of (A) oncocytoma and (B) acinic cell cancer. Compare the TEM micrographs *Figures 19 and 20.* (A 925×, B 925×).

As many of these structures have a real bearing on the function of the cells, and presumably also on their malignancy, a thorough knowledge and further information are of great interest. Indeed it may be that many of these features have their main significance as evidence of different grades of cellular activity.

Moreover the cytoplasm may contain structures of a specific nature which is of importance for the diagnosis of a tumour.

In cytological smears for light microscopic examination it may sometimes be very difficult in certain salivary gland tumours to determine whether the granulation of the cytoplasm in tumour cells is caused by e.g. serous granules, mitochondria or lysosomes, a judgement which alone decides whether it is e.g. an acinic cell cancer (Erlandsson & Tandler 1972), an oncocytoma (Tandler et al 1970) or a Warthin tumour (Tandler & Shipkey 1964) or a granular cell myoblastoma (Sobel et al 1972). The cells which seem alike in light microscopy (Fig. 18) prove to be completely different in TEM. In acinic cell cancer (Fig. 19) the cytoplasm displays large numbers of, in part, atypical densely clustered secretory granula of varying size. The oncocytoma cells show (Fig. 20) an accumulation of pathological mitochondria, and the granular cell myoblastoma a multitude of, inter alia, lysosomes.

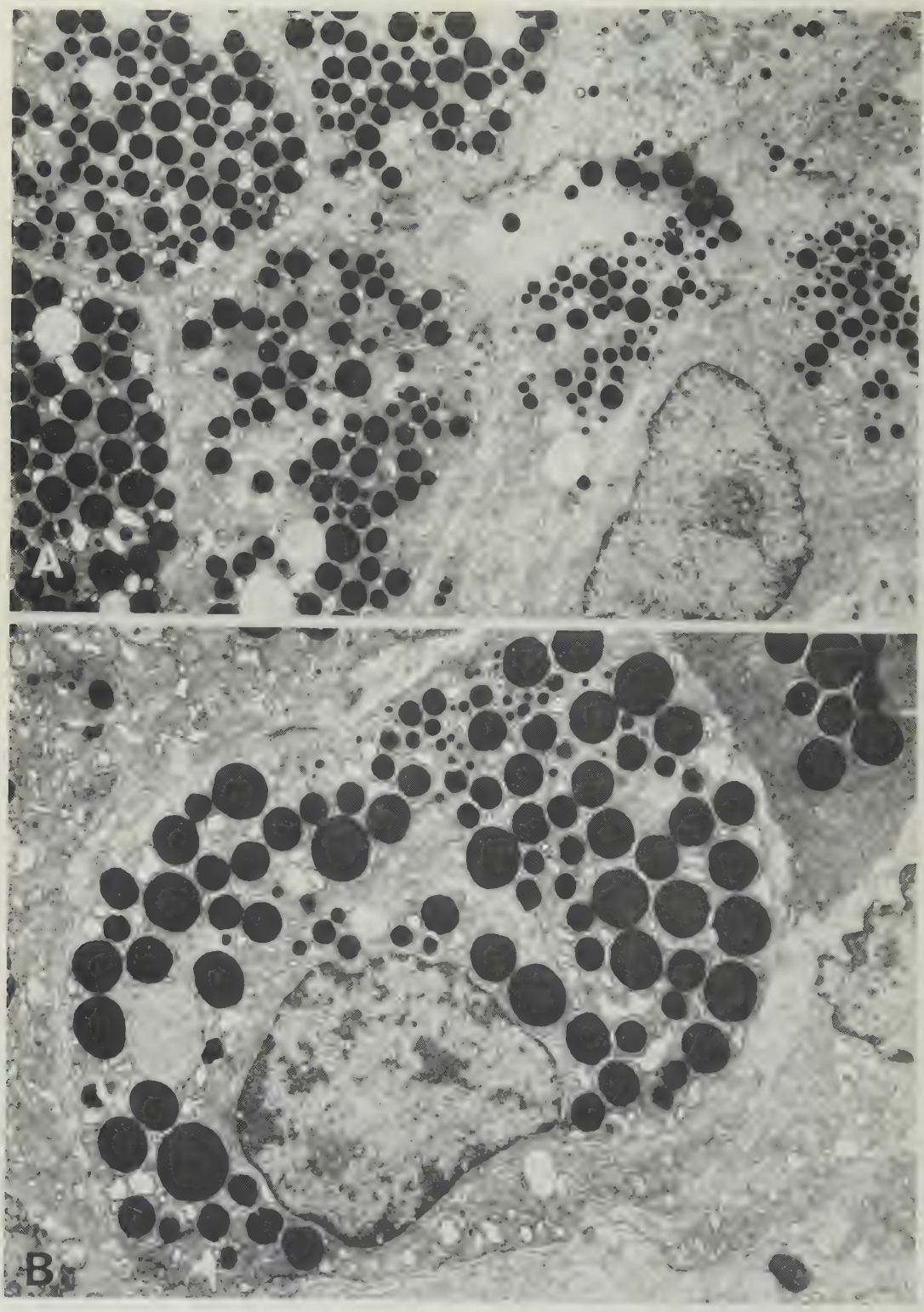


Fig. 19 A and B show secretory granules filling the cells in a case of acinic cell cancer. Many of the cells in this form of salivary gland tumour have a close resemblance to normal secretory cells. This is not at all the case in oncocytomas and adeno-lymphomas. Compare *Fig. 20* (A 6 500×, B 9 000×).

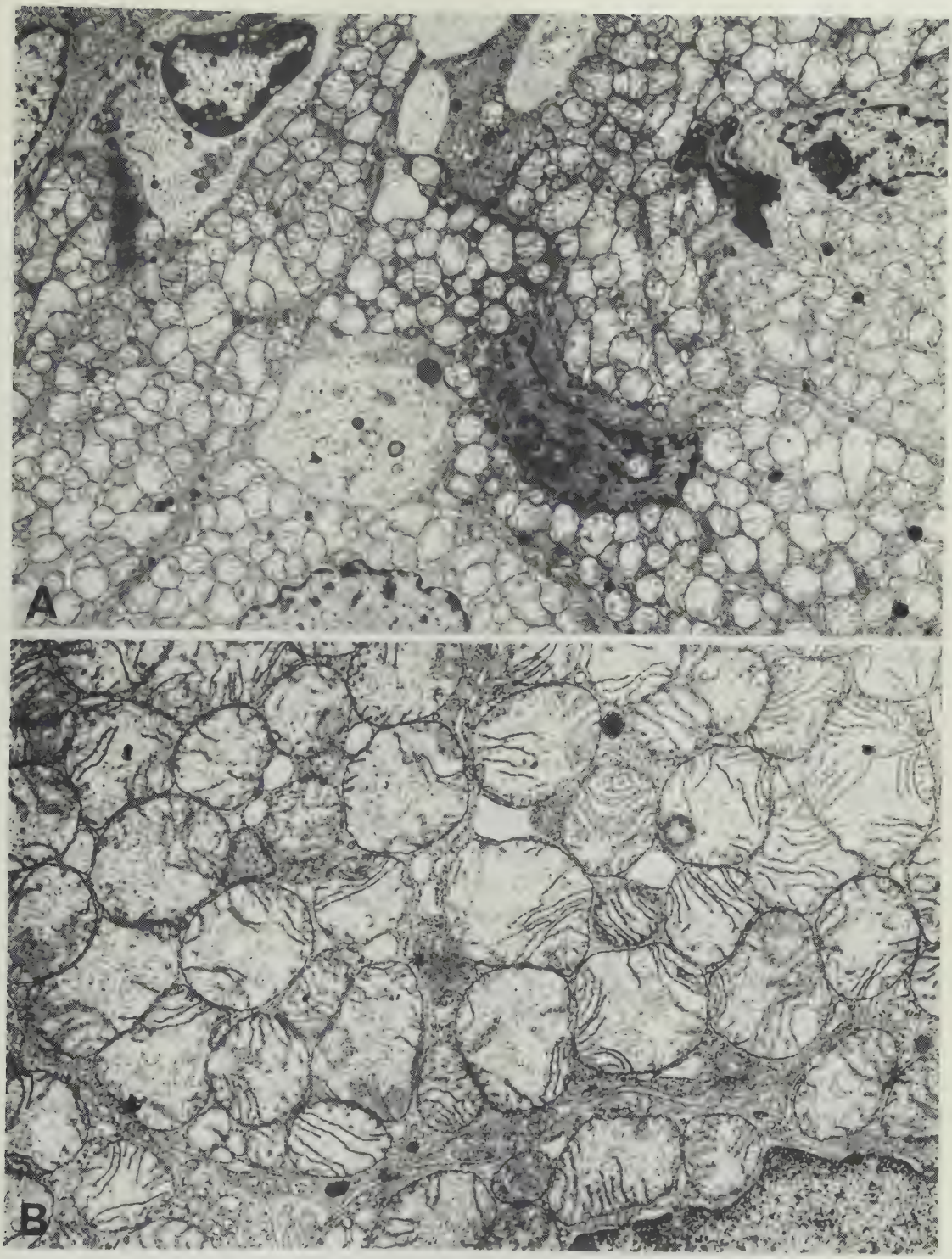


Fig. 20 A and B. Cells filled with pathological mitochondria in a case of oncocytoma. It is interesting to compare this case with the one shown in *Fig. 19* where the cells are packed with granules.
These two tumours are often difficult to differentiate on smears for light microscopy. (A 5500×, B 19000×).

Mitochondria in tumours studied by TEM can be observed in far greater detail. In some tumours the mitochondria may be almost normal in appearance, with regular borders and cristae. Other highly atypical and sometimes characteristic mitochondria were observed in our TEM aspirates. Various forms of atypical mitochondria are illustrated in Fig. 21. Figs. 21 B, C and D are particular interest since their appearance impelled us to suspect a renal cancer which was thus clinically diagnosed. These mitochondria reveal bizarre formations with their cristae often concentrically layered. Elongated, club-like, and dough-nut-shaped mitochondria were also seen in this case.

These findings in renal cancer are described by, inter alios, Seljelid and Ericsson (1956) and by Keyhani (1969), but are nevertheless not specific for this type of cancer. The case will be further discussed later.

In several cases the appearance of the mitochondria in tumours may not be characteristic, but merely show a deformation due to a degenerative process. Such mitochondria can be seen in Fig. 21 A. A similar type of structural modification has been observed in different kinds of tumour. These changes often appear in the form of myelin figures, which by some authors are regarded as a fixation artefact (Ericsson and Biberfeld 1967).

Some of the cytoplasmic inclusions may be highly characteristic and some can be observed by both light and electron microscopy, such as the Charcot-Leyden crystal in Fig. 22, or the eosinophilic granule in the same figure. The aspirate was taken from a skeletal destruction in the temporal region of a woman where both X-ray and cytological smears made the diagnosis of eosinophilic granuloma strongly suspect.

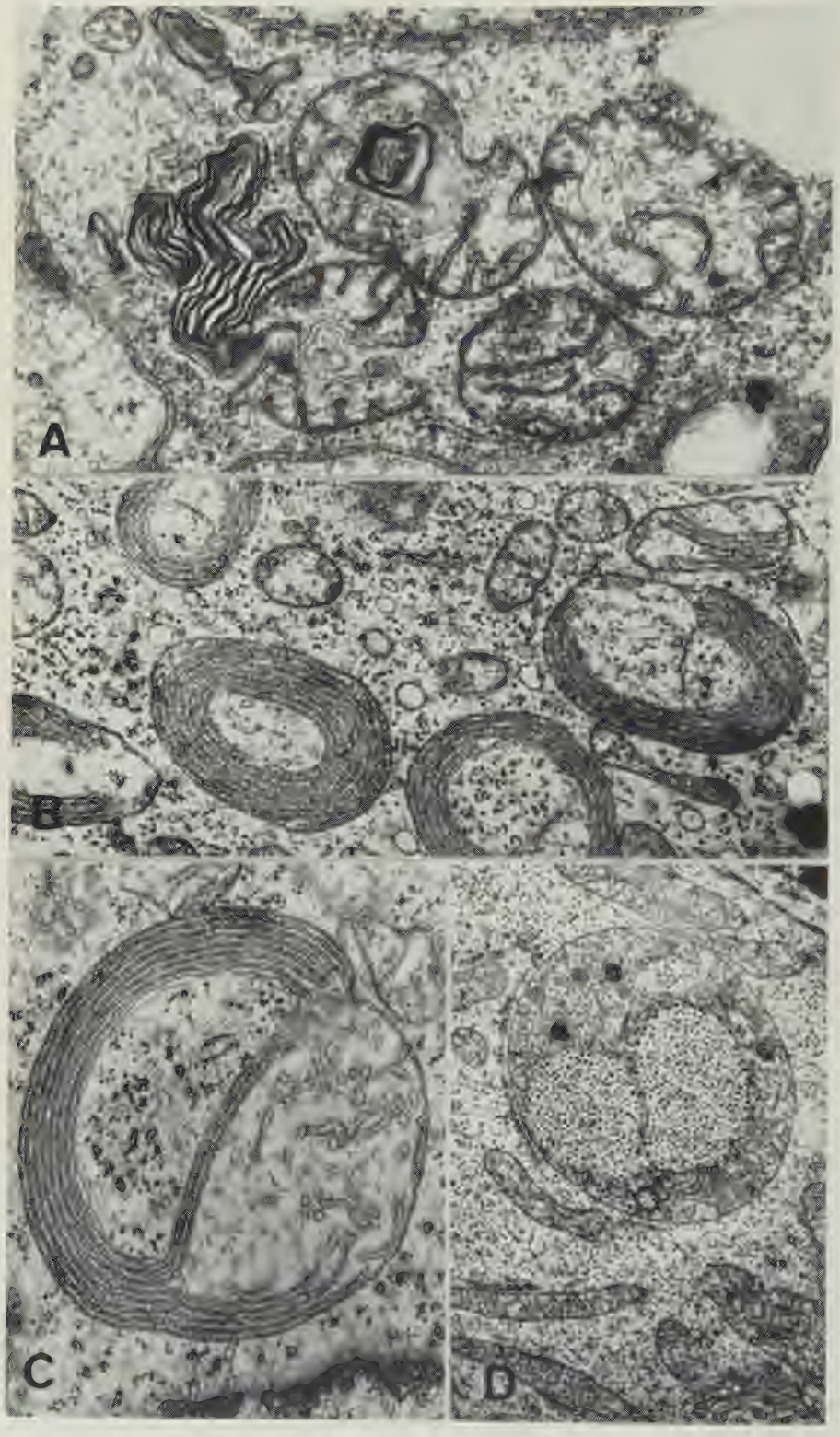
As a rule the pathologist or cytologist can reach his diagnosis with the help of routinely prepared slides for light microscopy as here. Difficulties may arise, however, with regard to poorly differentiated tumours. With the help of TEM it is at times possible to identify persisting intracytoplasmic structures as characteristic of the tissue of origin. The amelanotic malignant melanoma often difficult to diagnose is a tumour of this type.

TEM can disclose immature stages of the melanosomes in the form of premelanosomes (Melanosome stage II according to Fitzpatrick et al. 1971) which allows of confirmation of the diagnosis. Figures 23 A and B illustrate melanosomes of differing degrees of maturity in the form of, inter alia, premelanosomes, with their typical zig-zag pattern and more mature granules containing amorphous dark melanin (Melanosome stage IV). (Fig. 23 C). Other structures which are difficult to discern in light microscopy but have a characteristic picture in TEM are amyloid. Fig. 24 depicts aspirate from a lymph gland metastasis in the neck from a medullary thyroid cancer where the diagnosis could not be reached solely on the cytological smears. TEM allows clear perception of densely packed amyloid fibrils. In Fig. 24 A and B it is also possible to discern granules which are interpreted as being of endocrine nature. The calcitonine titer proved to be high in this patient. The diagnosis medullary thyroid cancer may therefore be regarded as certain.

The question of whether the salivary glands also exert endocrine activity was asked long ago, inter alios by Feyrter in 1961. One of our salivary gland tumours, which was primarily diagnosed from smears as an atypical pleomorphic adenoma (Fig. 2 D) and on histopathological determination classified as cancer in a pleomorphic adenoma, showed in TEM plentiful accumulations of relatively small granula (Fig. 25). They did not resemble ordinary secretory granules in appearance but provoked suspicion of endocrine activity. Silver staining according to Grimelius (1968) was positive, so that the TEM finding could be confirmed. The case is further studied and will published separately

A number of other, non-specific, cytoplasmic inclusions are illustrated in Fig. 26. Fig. 26 A shows a secondary lysosome/phagolysosome, and primary lysosomes and presumably lipid droplets.

Ordinary cytoplasmic inclusions are glycogen particles which, however in TEM may often be difficult to distinguish from polyribosomes, or even artefacts such as lead deposits. Fig. 26 B shows an abundance of glycogen in rosette formations, from a liver puncture. Fig. 21 C and D also reveal that the glycogen may be located inside a pathological mitochondrion in cases of suspected hypernephroma metastasis. Seljelid and Ericsson (1965) demonstrated this pronounced accumulation of glycogen in the mitochondria in renal cancer. Nevertheless already in 1964 Tandler and Shipkey showed that the same glycogen incorporation can be seen in the mitochondria in Warthin tumours. Similar mitochondrial changes can also be perceived in a large number of myopathies.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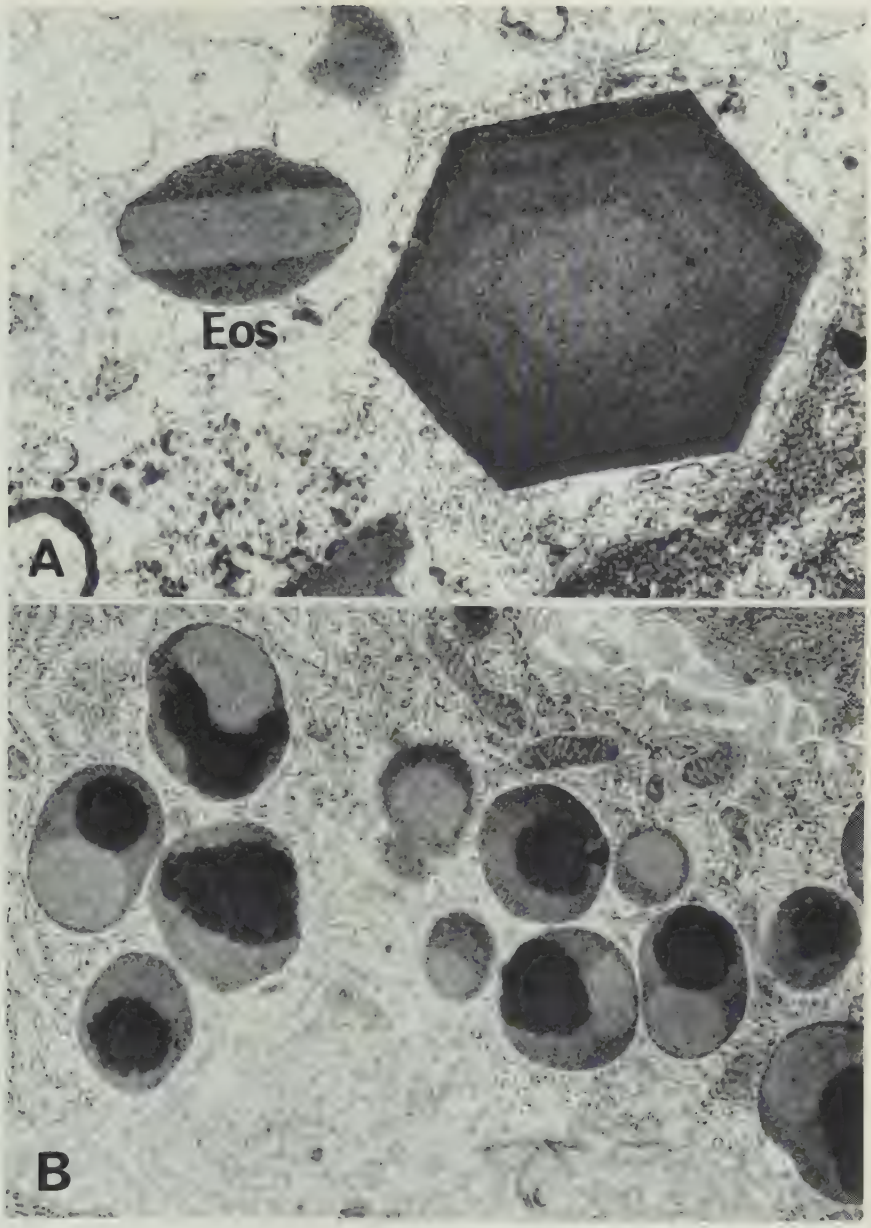


Fig. 22 A shows a TEM-micrograph from a case of eosinophilic granuloma in the temporal bone with an eosinophilic granule (*Eos*) and a Charcot-Leyden crystal. (37 500 ×). B shows mucous granules in the submaxillary gland of a patient with sialo adenitis. (17 500 ×).

Fig. 21 Atypical or pathological mitochondria from: A a case of malignant lympho-epithelial lesion and B, C and D a case of multiple salivary gland tumour. This case was histopathologically classified as "acinic cell cancer". *Against the background of the form and appearance of the mitochondria as found by TEM on an aspiration from the parotid gland, a renal tumour was suspected and X-ray of the kidneys showed bilateral renal tumours. This was also verified by cytologic aspirate.*
(A = 45 500 ×, B = 25 000 ×, C = 30 000 ×, D = 14 000 ×).

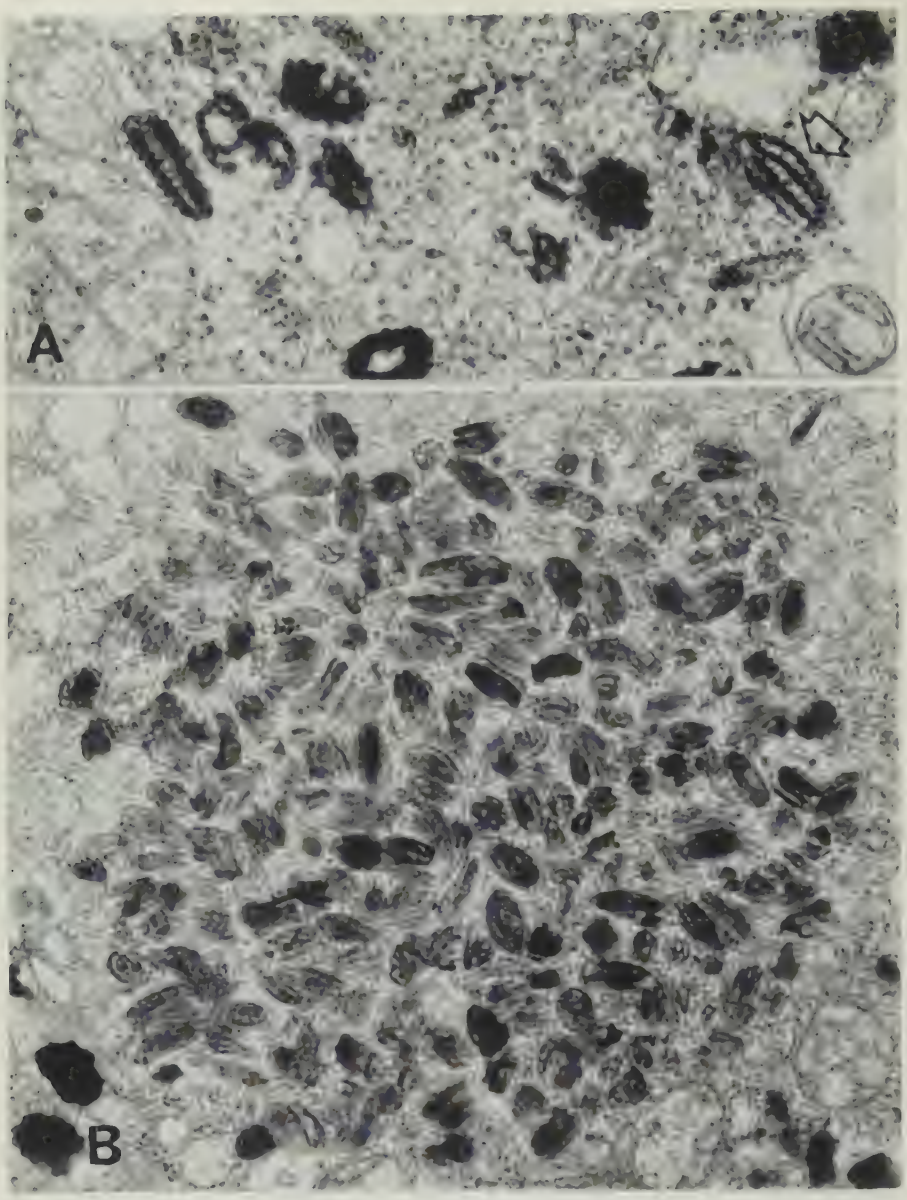


Fig. 23 TEM-micrographs from a lymph node metastasis of malignant melanoma.
A. Premelanosomes (arrow) with typical "zig-zag" arrangement, (80000×).
B. Agglomeration of premelanosomes and melanosomes, (32500×).

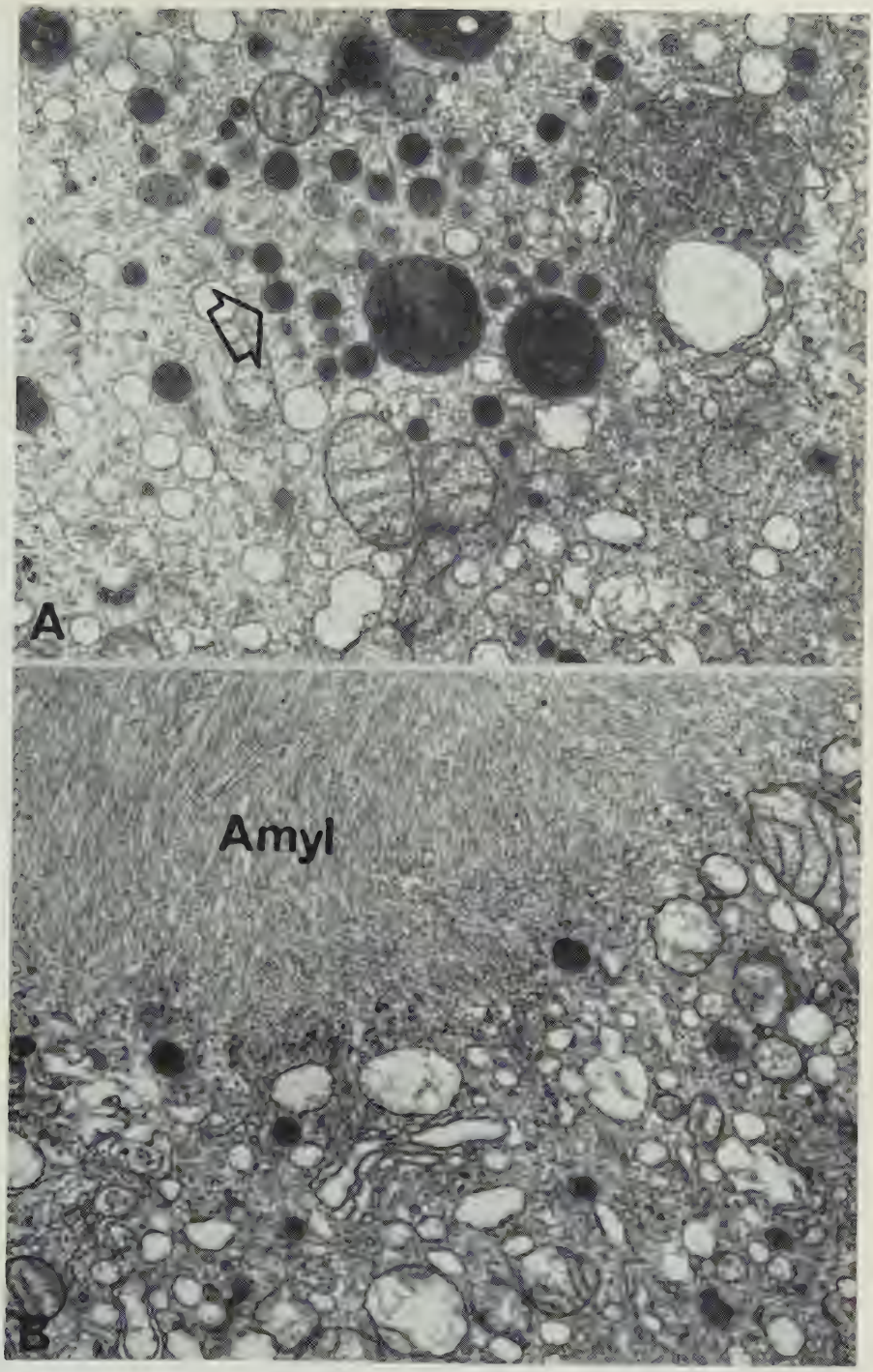


Fig. 24 These two micrographs are from a case of medullary thyroid carcinoma. In A cytoplasmic inclusions (arrow) have been supposed to be evidence of endocrine production, (32500×). In B typical amyloid (*Amyl*) can be seen close to or in a cell with much deformed cytoplasm, (29000×).

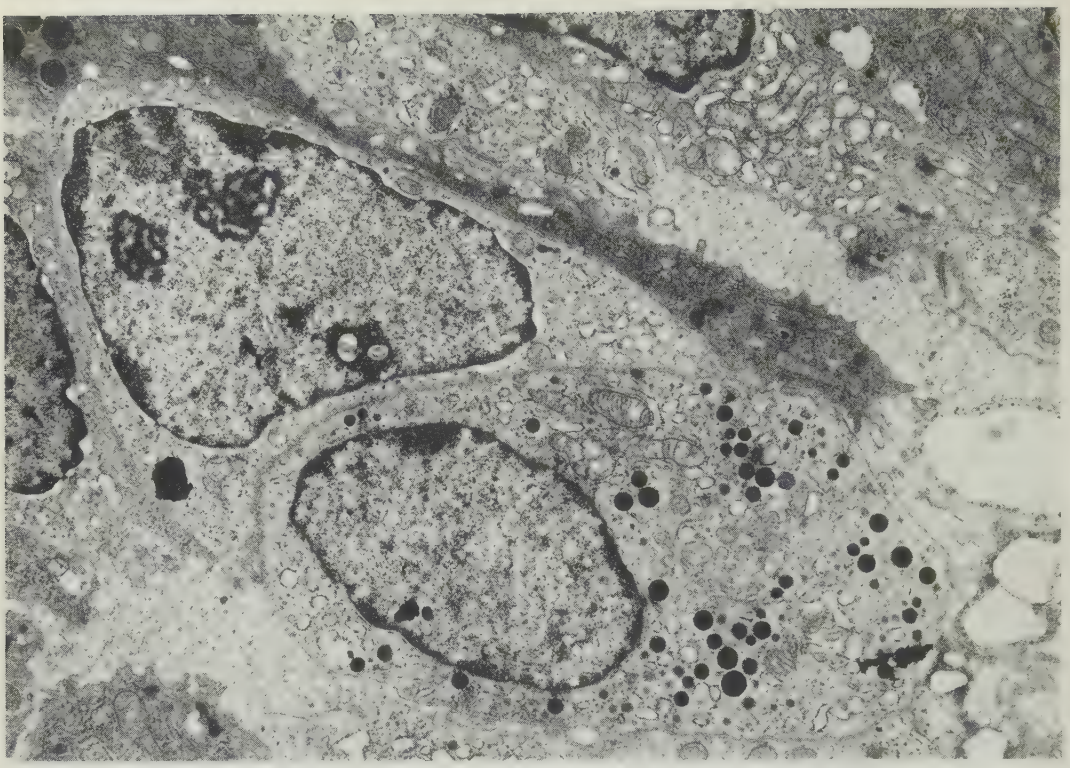
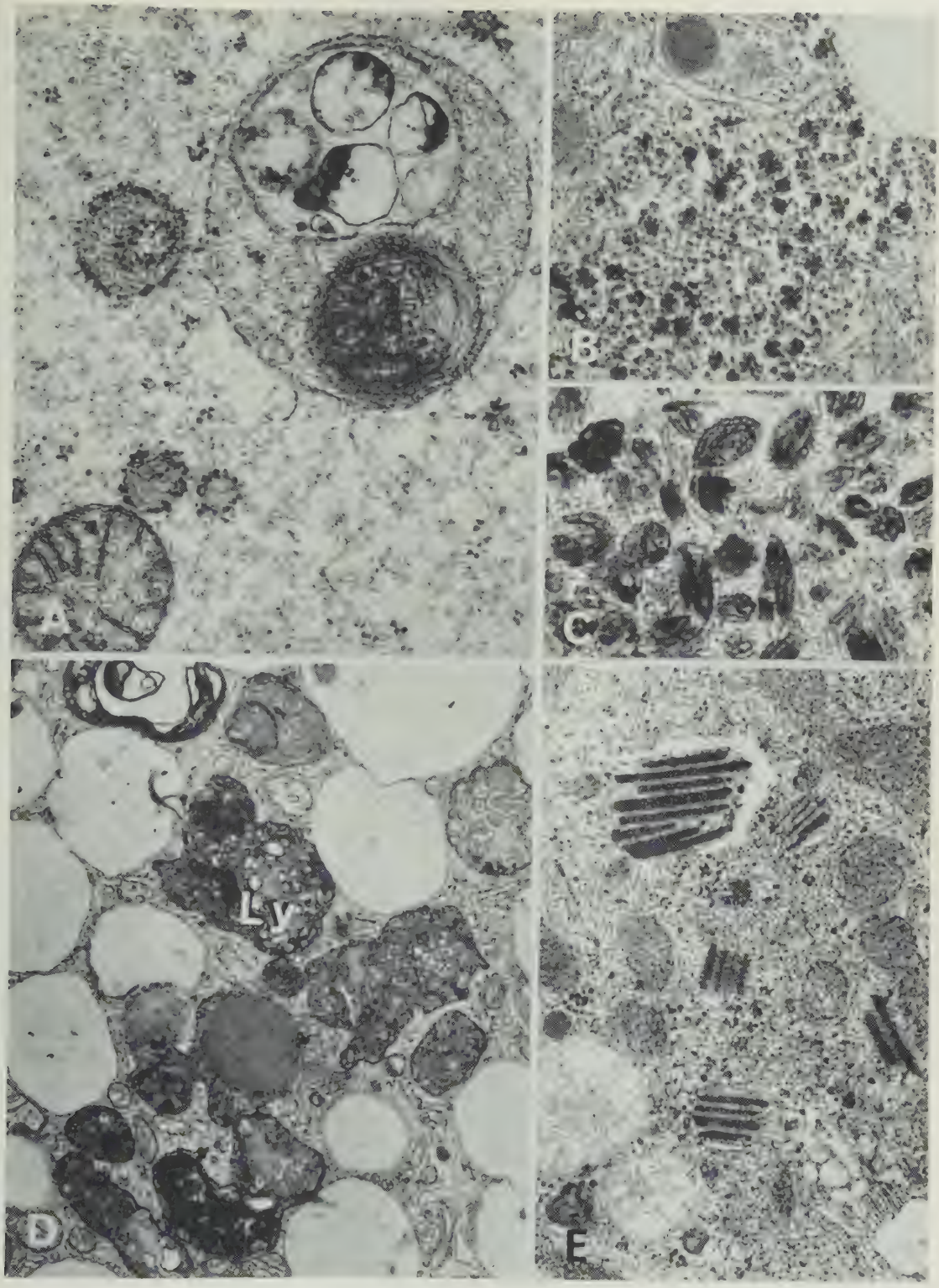


Fig. 25 Granules of endocrine origin in a tumour of the parotid gland. The tumour was interpreted as a poorly differentiated cancer, but specific staining (Grimelius) demonstrated the nature of the granules. (4500×).

Fig. 26 Micrographs showing different types of cytoplasmic inclusions.
A. Phagolysome containing partly destroyed cell structures in a case of pleomorphic adenoma, (45 500×).
B. Glycogen granules in an enlarged salivary gland, presumably sialosis.
C. Melanosomes from a case of malignant melanoma, (45 500×).
D. Agglomeration of lysosomes (*Ly*) and lipid droplets in a case of partly necrotic adeno-papillary cancer in the parotid gland, (17000×).
E. Pigment or crystalloid structures in a case of poorly differentiated parotid tumour (malignant melanoma?), (40000×).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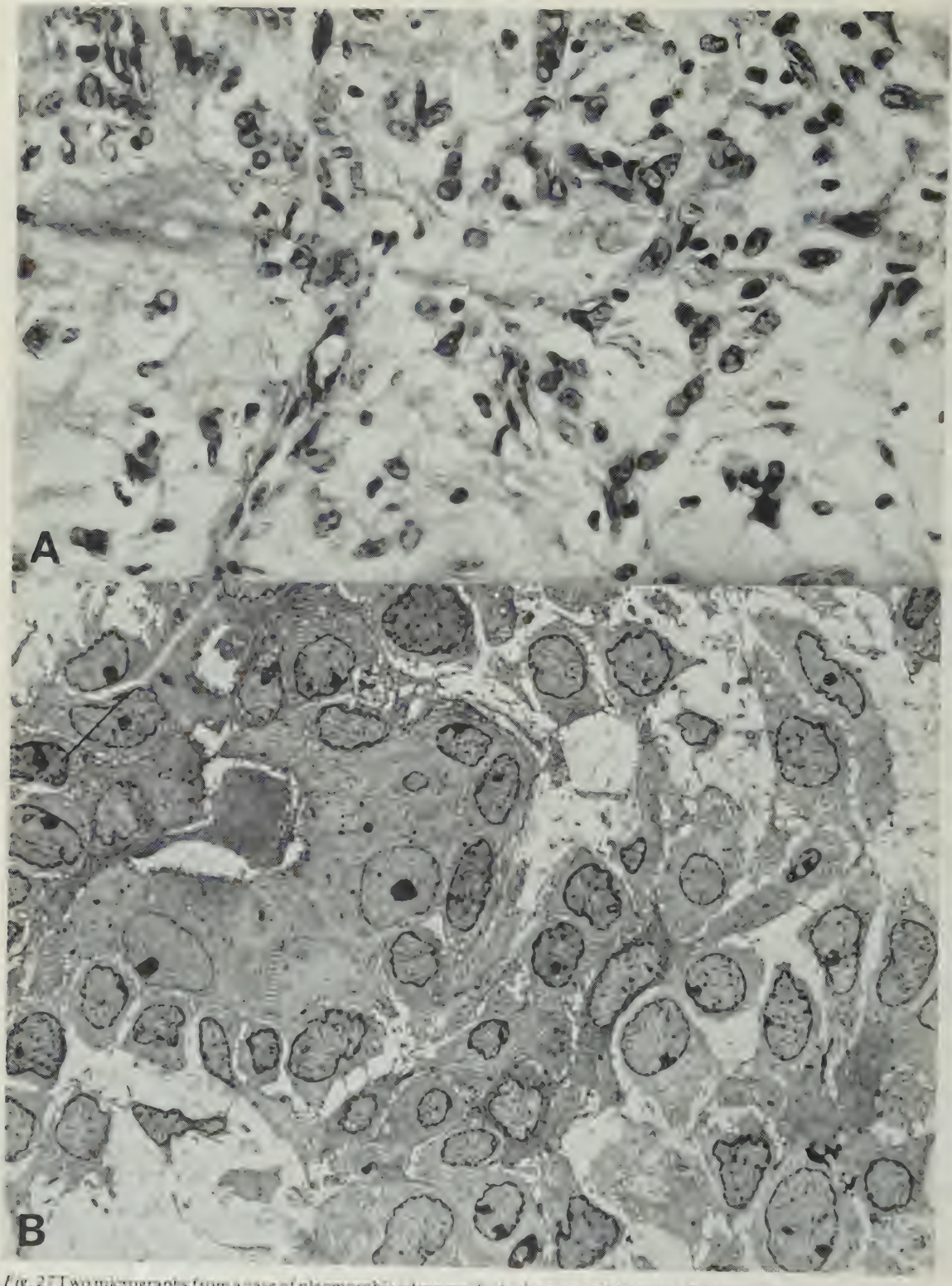


Fig. 27. Two micrographs from a case of pleomorphic adenoma. In A, the cyto-architecture of the tumour is seen in a specimen taken at the operation. In B, the TEM micrograph from an aspirate in the same case gives a picture showing good agreement. (A. 200×, B. 1 800×).

Part III. A STUDY OF SOME CASES OF ADENOID CYSTIC CANCER

INTRODUCTION

Tumours of the salivary glands are sometimes difficult to diagnose because they display highly variable histological structures. Their classification therefore remained obscure for many years. The new WHO classification was established in 1972.

Adenoid cystic cancer (ACC) has long been controversial and difficult to classify. First described by Billroth in 1859 it was then called cylindroma, a name based on a structural description. This name was later used also for more benign types of tumour, such as hidradenoma and various forms of basal cell cancer. In 1930 Spies introduced the term ACC, which was not generally accepted until 1954, in connection with Foote and Frazell's classification of salivary gland tumours. Thackray and Lucas (1974) gave a detailed description of the many variants of ACC, from the most typical stroma-rich types with cystic or cribriform structures to the more solid manifestations with indeterminate structures (Fig. 28).

The pre-operative diagnosis of salivary gland tumours is important for the planning of an operation. This applies not least to ACC, which was regarded for many years as a comparatively benign tumour, by reason of the usually good five year survival rate. A carefully analysis of ACC shows, however, that it is possible to distinguish varying degrees of malignancy. Thus investigations by Eneroth and Hjertman 1966, Blanck et al. (1967), Eneroth (1970, 1976) and Blanck (1976) demonstrated that it can be highly malignant, especially when appearing predominantly solid in structure. Knowledge of the diagnosis of the tumour is of special interest for the planning of surgery, considering furthermore that according to certain authors (Blanck et al. 1967) ACC is radiation sensitive, and that radiotherapy should be undertaken pre-operatively.

For pre-operative diagnosis various oncologists have resorted to coarse needle biopsies ad modum Silvermann (1938), and surgery biopsies. But these biopsy techniques proved to involve a risk of local metastasis by implantation, which may complicate subsequent surgery and increase the possibility of relapse. Biopsy by means of fine needle aspiration, however, proved to involve very little risk in this respect (Engzell et al. 1971). Using a comprehensive material these authors could demonstrate that the risk of metastasis after fine needle biopsy was negligible. Moreover the patients examined included 157 cases of pleomorphic adenoma (Fig. 27), followed up for 10 years after puncture. In no case was it possible to prove that the puncture gave rise to dissemination of tumour cells. Three relapses which were observed were thought to result from non-radical primary surgery. Fine needle biopsy for pre-operative clinical diagnosis of tumours has, as already pointed out, been in use in Scandinavia for many years. Studies on the salivary gland include Berge and Söderström (1963), Eneroth and Zajiceck (1965, 1966), Mavec et al. (1964), Eneroth et al. (1967 and 1971), and Zajiceck (1974).

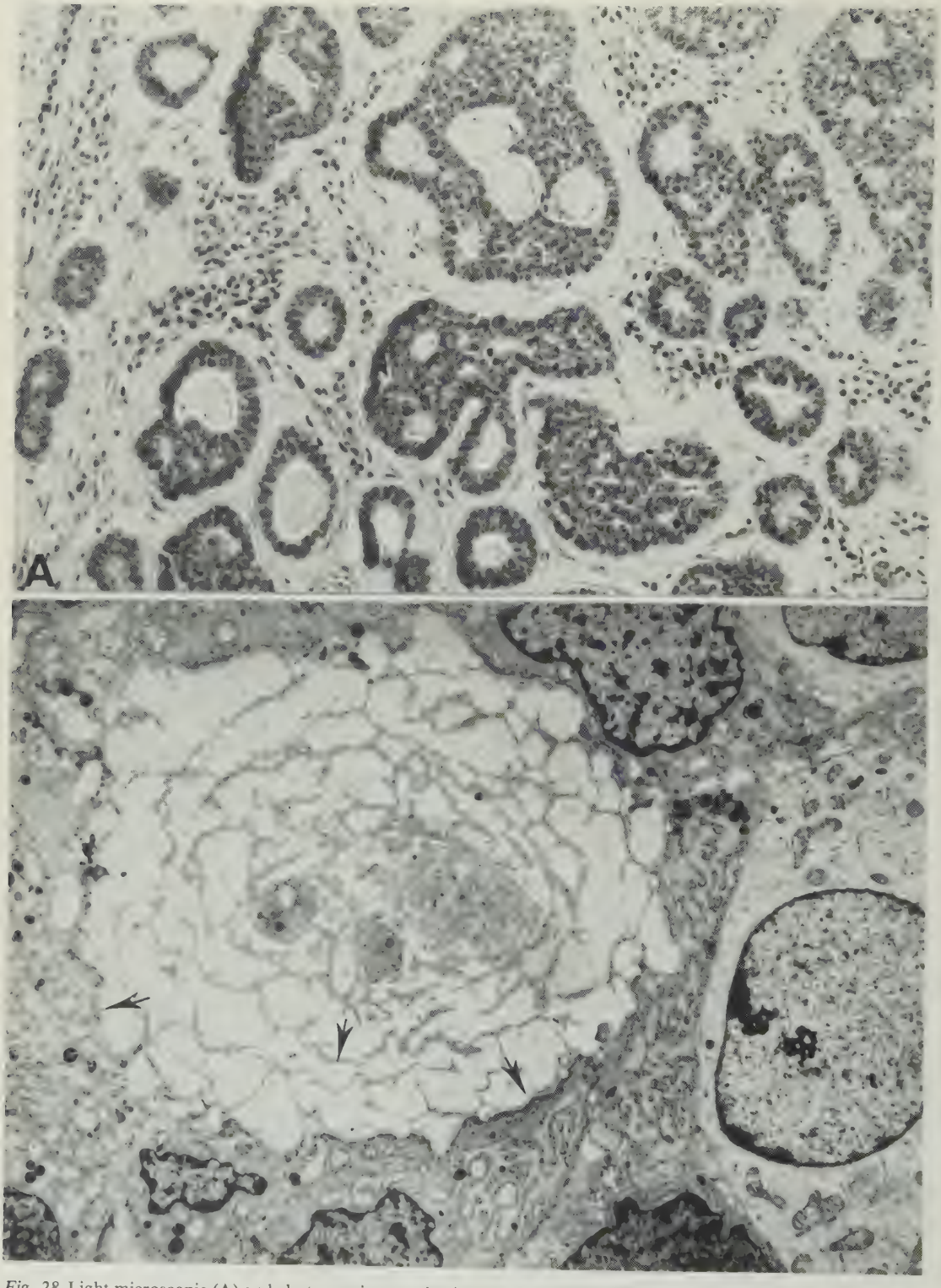


Fig. 28 Light microscopic (A) and electron microscopic picture (B) from a case of adenoid cystic cancer (ACC). In A the typical cribriform appearance of the tumour is evident, and in B the structure of a pseudocysts (*PC*) can be seen. Inside the pseudocysts there are several basement membrane-like layers (arrow) and also some cell remnants. (A 200×, B 6300×).

Only a few papers have been published on cytological diagnosis of ACC. One of the more extensive was carried out by Eneroth and Zajiceck (1969), who showed that in 25 of 45 cases of ACC diagnosis could be made primarily on the incidence of well defined "spherical bodies" or "mucoid globules" which in May-Grünewald-Giemsa (MGG) staining took on a dark red colour. These structures were chiefly seen when the tumour was cribriform in its mode of growth. Furthermore, they considered that in such cases the diagnosis was easy to make by cytology. When the tumour is of the more solid type it is more difficult to reach the correct diagnosis.

According to several authors fine needle aspiration biopsy in the hands of an experienced cytologist gives just as representative a material as coarse needle biopsy and surgery biopsies without their disadvantages (Zajiceck 1974 and Eneroth 1976). Moreover surgery biopsy from salivary gland tumours is considered by many to be a contra-indication, because of the risk of implantation.

Nevertheless we must admit that it is sometimes difficult or impossible to give a diagnosis even when aspirated material is sufficient and frozen sections are done as well. It might even be difficult to determine whether the tumour is benign or malignant. Therefore it is of great interest to increase our knowledge of these tumours.

The introduction of electron microscopy has given further essential information on the inner structure of tumour cells, and certain organelles have been found to be specific for some tumours (Ghadially 1975). These TEM investigations were carried out mainly on surgery material.

On basis of the experience gained with TEM in tumour biopsies, Hagelqvist and Engström (1973) made an electron microscopic study of material obtained by fine needle aspiration biopsy, and demonstrated that TEM can be performed with qualitatively good results in salivary gland tumours. These preliminary studies showed that the method would be a valuable supplement to the pre-operative diagnostics when the conventional cytological diagnosis was doubtful or insufficient. Certain salivary gland tumours may have a similar cytological appearance, whereas TEM examination may reveal different intracellular structures of importance for differential diagnosis. This is the case e.g. in decision on the diagnosis acinic cell cancer contra oncocytoma, which may closely resemble each other cytologically. Ultrastructurally it is easily shown that large numbers of atypical mitochondria occur in oncocytoma, while in acinic cell cancer there are large numbers of granules in the cytoplasm. Similarly, it would be of great interest to find specific structural differences which could permit a more accurate delimitation of ACC from other types of tumours, e.g. atypical pleomorphic adenoma. Although TEM examination would not allow a clear differential diagnosis, it is of the greatest importance to collect as much information as possible on the structure of tumours.

Ultrastructural studies of ACC performed on material obtained by surgery have been available for over 10 years, and have given valuable knowledge of the structures from which the tumours arise (Foote and Frazell 1954, Market 1965, Friborsky 1966, Eneroth et al. 1968,Hübner et al. 1969, Hoshino and Yamamoto 1970, Hamperl 1970, Tandler 1971, Engström and Stahle 1972 and Chisholm et al. 1975). Most authors hold that ACC, like presumably pleomorphic adenoma (PA), originate from

the terminal ducts of the salivary gland, specifically, the intercalated ducts. The majority also consider that the myoepithelial cells play a vital role. Both Eneroth et al. (1968) and Tandler et al. (1971) have also studied the cystic structures which are found in the cribriform type of ACC, and believe them to contain material resembling a basement membrane. According to Hübner et al. (1969), and Eneroth (1968), these fibrillar structures are of aperiodic type, Tandler is, however, of the opinion that the content of "the pseudocysts" manifests a certain periodicity. In the context of today's debate on the structure of the basement membrane, the question of its real structure should in all probability be studied more thoroughly, (Trelstad 1977).

In 1975 Chisholm et al. carried out a qualitative and quantitative electron microscopic investigation of ACC in "small salivary glands". They were able to confirm that the cyst-like cavities which give the tumour its characteristic cribriform appearence contain "replicated" basement membrane-like material. Using a stereological investigation technique they demonstrated furthermore that the cells designated ductal in the tumour occupied 75% of the volume, myoepithelial cells 3%, and acinar cells 2%, which contradicts the results of most earlier researchers, who asserted that the myoepithelial cells predominate (Bauer and Fox 1945, Friborsky 1963, Thackray and Lucas 1963, Hübner et al. 1969). Thus opinions on the histogenesis of ACC are still divided, and justify further study.

Within the framework of the investigation on TEM and aspiration cytology in head and neck tumours, we wished in this investigation to illustrate the ultrastructure of 13 cytologically certain, or highly suspect, adenoid cystic cancers, and thereby compare aspiration biopsy and surgery material in terms of both quality and quantity. Moreover there is the question if TEM examination of aspirates can yield more diagnostic information, than is attained by conventional cytology, which can make the pre-operative diagnosis more certain.

A. MATERIAL

The material consists of tumours, in which the pre-operative diagnosis ACC was regarded as certain (8 cases) or highly suspect (5 cases), when evaluated by conventional cytological methods. Fine needle aspirates were collected for TEM examination from all these tumours. Further specimens were taken post-operatively in all 13 cases for light microscopy, and in 11 for electron microscopy, and compared with the pre-operative aspirates. In seven of these cases we also performed a semi- quantitative comparison between the cell populations in material obtained pre- operatively from aspirates and after operation by biopsy. The semi-quantitation was done on TEM micrographs.

A survey of the material is given in Table III, which also illustrates agreement or disagreement in the classification of tumours by different techniques.

CASE NO	ORIGIN	DIAGNOSIS		TEM		SEMI-THIN SECTION FINE-N.P.	REMARKS
		FINE-N. PCT.	HISTOPATH.	Aspirates	Biopsy		
EH 163	Tongue base	ACC	ACC	+	—	+	
EH 164	Palate	ACC	ACC	+	—	+	
EH 167	Lgl met. from parotid gland	ACC	ACC	+	+	—	
EH 214	Lgl met from larynx	ACC	ACC	+	+	+	
EH 220	Parotid gland	ACC	ACC	+	+	+	
EH 225	Parotid gland	ACC	ACC	+	+	+	
EH 249	Parotid gland	ACC	ACC	+	+	+	X-ray 4000 rad
EH 255	Parotid gland	ACC	ACC	+	+	+	X-ray 4000 rad
EH 223	Submand. gland	Highly suspect ACC	ACC	+	+	+	
EH 230	Palate	Atypical PA alt ACC	ACC	+	+	(+)	Frozen section D: PA
EH 237	Parotid gland	Atypical PA alt ACC	ACC	+	+	(+)	Frozen section D:PA alt Mucoep. ca
EH 182	Palate	Highly suspect ACC	Atypical monom. adenoma	+	+	+	
EH 206	Parotid gland	Atypical PA alt ACC	Monomorphic adenoma	+	+	+	

Table III. **Survey of material included with diagnosis as evaluated from fine needle aspiration cytology and biopsy.**

Abbreviations: ACC-adenoid cystic cancer,; PA- pleomorphic adenoma; Monom.ad.-monomorphic adenoma Mucoep.ca — mucoepidermoid cancer.

This survey suggests the presence of a discrepancy in the diagnosis of the tumours by means of the methods used. To simplify the problem for discussion the material can be summarized as in Table IV.

Table IV

Agreement	cytology (ACC) — histopat. (ACC)	6
Highly suspect	cytology (ACC) — histopat. (ACC)	3
Highly suspect	cytology (ACC) — histopat. (other diagnosis)	2
X-ray cases	cytology (ACC) — histopat. (ACC)	2
	Total	13

B. METHODS

The material was used for diagnostic purposes as indicated in the following survey.

a) **Fine-needle aspirates**

1. For light microscopy material was aspirated, smeared on object glasses, air dried and stained by MGG.

2. For electron microscopy (TEM) material was aspirated, fixed and embedded in Epon 812 as described in Part I D.a.

b) **Biopsy material** (taken at operation)

1. For light microscopy, a thin slice of tumour was fixed in formaldehyde (Histofix*), embedded in paraffin, sectioned and stained in Hematoxylin-Eosin, PAS, Alcian-green and Congo-red.

2. For TEM, small pieces of tumour material were collected, fixed, and embedded in Epon 812 as descibed in Part I D.a.

* Histofix (Formaldehyde in cacodylate buffer)

c) For a **semi-quantitative comparison** Epon-embedded material for TEM from operation material (B) and aspirates (A) were studied. The aim was to make a pilot study of the tumour cell content in the two types.

The material for semi-quantitative comparison consists of the 7 cases shown in Tables III and IV. The cases chosen were:

3 cases with complete agreement (ACC) between cytology and histopathology (EH 167, EH 214, EH 225).

2 cases highly suspect to be ACC cytologically and clearly described as ACC histopathologically (EH 230, EH237).

2 cases highly suspect cytologically but both described histopathologically as monomorphic adenomas (EH 184, EH 206).

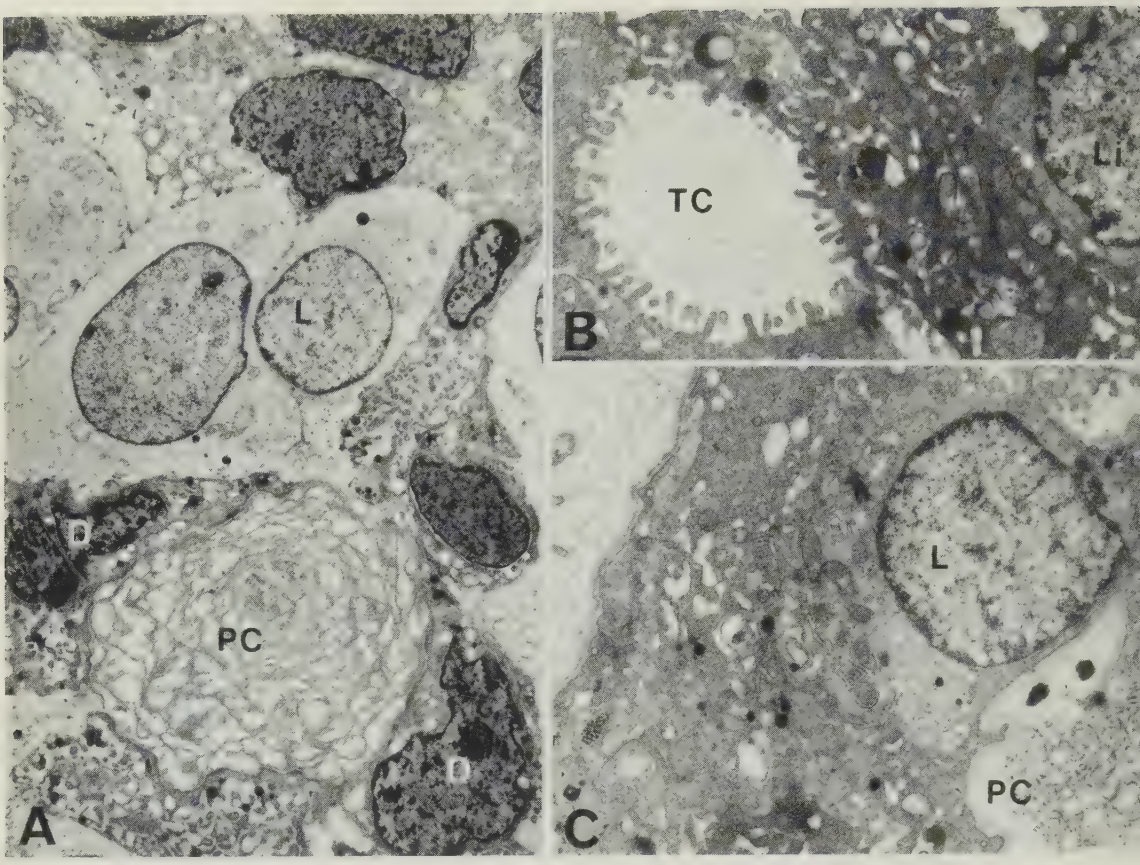
In this material 200 neighbouring cells chosen at random among TEM-sections from aspirates and biopsy specimens from every case have been evaluated, counted and compared. The classification of the cells was performed partly in accordance with definitions given by Chisholm (1974) and Hoshino and Yamamoto (1970). As criteria for the present classification we have chosen the following principles where the cells are designated as, L, L_i, D, D_i and Sq cells.

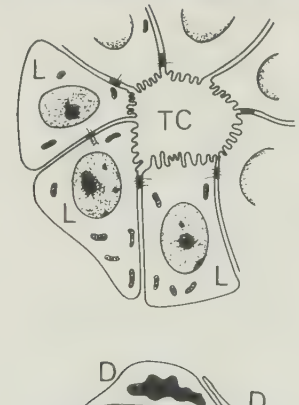
L-cells (light cells) correspond to the "duct-type cells" described by Chisholm et al. (1974). Hoshino and Yamamoto (1970) called them B-cells or "secretory type cells". The L-cells are large, ovoid or rounded. Their nuclei are often round but may be slightly serrated or cleft. The chromatin is very finely granular with condensations towards the nuclear membrane. There are often 1—2 large nucleoli, frequently with a distinct reticular nucleolonema. The cells contain but few cytoplasmic organelles in the form of mitochondria and endoplasmic reticulum. The ribosomes often appear in rosette-formations, polyribosomes. The cells are interconnected by an abundance of desmosomes. Microvilli can be observed where the cells are arranged along luminal surfaces. Neighbouring cells often have interposed intercellular material although they are richly interconnected by the desmosomes.

D-cells (dark cells) correspond to the "myoepithelial cells" described by Chisholm et al. and to the A-cells as these are classified by Hoshino and Yamamoto. The structure of these cells is in many ways reminiscent of normal myoepithelial cells. They are dark in TEM-micrographs, often irregularly oblong or stellate, and smaller than the L-cells. The chromatin is abundant and densely distributed throughout the nucleus which usually has an irregular form. The cells have a rich endoplasmic reticulum and more mitochondria.

L_i- and D_i-cells correspond to the "transitional forms" described by Hoshino and Yamamoto. Similar cells were seen in pleomorphic adenomas by Eneroth and Wersäll (1966) and called intermediate forms or incompletely differentiated duct cells. They were also described as intermediate types by Welsh and Meyer (1968) and given various names by other authors.

L_i-cells (light intermediate cells) have a cytoplasm as in the L-cells but the nuclear membrane is irregular and the nuclear chromatin more clustered than in the L-cells.





PC = Pseudo-cyst
Cyst-like cavities delimited by structures resemblin
basal membrane. The cavities are normally filled wit
mucus-like material

TC = True cyst
The cyst walls are usually delimited by ''duct-type''
cells or L-cells with microvilli along the luminal bo
der

L-cells — light cells ''duct-type'' cells.

D-cells — dark cells

L_i- and D_i-cells — intermediate cells.

Fig. 29 Three TEM- aspirate micrographs from ACC-tumours showing light and dark cells, true cyst and pseudocyst. The schematic drawing explains the principles in the arrangement of the cells and their organelles. (A 3 500×, B 7 000×, C 7 000×).

D_i-cells (dark intermediate cells) have a rather dense irregular cytoplasm and a fairly well developed endoplasmic reticulum with mitochondria but the nuclei are ovoid, not as irregular and serrated as in typical D-cells.

Desmosomes are not observed only in D-cells but may appear between L- and D-cells and also in the intermediate forms. They are most frequent between L-cells.

Some authors have defined the cells in ACC as either ductal or secretory. The present classification is used to avoid such a definition as it is not possible in our material to ascertain the original cell type using TEM alone. It is so well known that the cytoplasm and cell nuclei in rapidly developing cell cultures or tumours may change both nuclear form and cytoplasmic density during mitosis. Density of nuclei and cytoplasm alone can, however, be used in the comparison of cell content from two forms of biopsy in the same tumour.

This context calls for a further definition of the so-called *pseudocysts* (PC), (Azzopardi & Smith 1959). This term denotes a cyst-like formation, outwards bordered by the plasma membrane of surrounding cells. Below the plasma membrane a basement-like, fine fibrillar, continuous membrane surrounds the pseudocyst. Several similar fine-fibrillar membranes are often seen inside the pseudocysts which may also contain structures resembling cell debris. Naked pyknotic nuclei can occasionally be observed in these formations. The thickness of the "basement membranes" is not uniform. The "true cysts" (TC), which may sometimes be ducts, consist of circumscribed cavities bordered by the L-cells which usually have microvilli on the surface facing the cysts (Fig. 29).

C. RESULTS

a) **Cytology and PAD in complete agreement.** ACC — ACC
EH 163, 164, 167, 214, 220, 225, 249, 255.

In these cases of complete agreement **the cytological smears** for light microscopic examination were rich in cells and contained numerous distinct, clearly delimited, circular or oval, mucous globules of different sizes. Moreover the scrutiny revealed patches of reddish-violet, homogeneous, and thread-like mucus of the same type as seen in the cysts, forming short strands between and outside the cell clusters. Where the cells were lying in isolation, open to classification, they consisted partly of large, light, vesicular cells (L-cells), partly of smaller, darker cells (D-cells). The cells inside the more compact parts of the tumour could not be classified. Around the globules the inner cell layer comprised light cells rather than dark cells although usually a mixture.

Corresponding histopathology revealed a tumour tissue dominated by cribriform structures with cysts of varying size and shape. In one case, however, the solid areas

predominated. In another, the incidence of mucus was surprisingly high, and apart from the typical cribriform structures the cysts formed elongated, ramified "duct systems" of the type often observed in ACC. The cyst-like structures were surrounded as a rule by one or more cell layer. The cells bordering the cysts were difficult to classify but dominated by L rather than D-cells. The cells were usually mixed in those cases where a differentiation was possible. Where staining of the mucus with Alcian green was carried out, the content of the cysts and the tissue between the epithelial cells were fairly heavily stained. The corresponding areas were only slightly stained when PAS was used. Congo red staining with regard to amyloid was negative in all cases but one (EH 164) in which it was strongly positive. (In this case Alcian green was only weakly positive and PAS negative).

At the corresponding ultrastructural examination fine needle aspirate revealed, apart from solid areas, an abundance of large and small pseudocysts (PC), all of which were delimited by a distinct, continuous line resembling a basal membrane. The PC were surrounded by equal numbers of L- and D-cells, the two often being intermingled. Many of the cells displayed the characteristics of the L_i and D_i types, so that the nuclei were dark in colour and resembled D-cells, whereas the cytoplasm had the criteria of the L or ductal cells. Similarly, the nuclei could be light, of the L-cell type while the cytoplasm was sparse and indented, reminiscent of the D-cells in this respect. Tonofilament-like structures were frequently discerned in the cytoplasm, presumably an expression of squamous epithelial metaplasia.

In the more solid areas the dense cell clusters often appeared to be composed of large, light L-cells with rounded, partly serrated nuclei, which were usually linked together by tightly packed desmosomes. Desmosomes were much less frequent in D-cells and intermediate forms.

The D-cells were easy to identify when they were typical, i.e. resembled myoepithelial cells with elongated, dark, often serrated nuclei and sparse cytoplasm containing filaments. D-cells were very often found in the form of intermediate cells. Similarly, a cell type suggesting metaplastic transition to squamous epithelium was often observed.

The D-cells were not as frequently found in large, compact clusters but lay in isolation, loosely linked with each other.

Almost every specimen displayed "true" lumina (TC) which were not delimited by a membrane, but an abundance of microvilli projected into the lumina. The boundary cells here consisted exclusively of the L type.

The following TEM findings are also worthy of mention:
One of the cases (EH 164) which was strongly positive to Congo red stain with regard to amyloid proved in TEM to consist chiefly of light L-cells, many of which contained small granules of obscure origin. Since an endocrine origin could not be excluded specimens from this case were stained with silver according to Grimelius (1968) and was positive.

One of our cases (EH 255) manifested a typical ACC when observed in the light microscope in both the cytologic smear and the semi-thin section. The TEM study

showed both L- and D-cells and pseudocysts but also a large number of cells with a very rich agglomeration of atypical mitochondria of the oncocytoma-like type. In this case no biopsy was made until the final operation, which took place after irradiation of the tumour. The combination of L- and D-cells with oncocytoid cells has not been observed in any other of our ACC-cases.

In conclusion it can be summarized that in these eight cases the light-and electron microscopic study revealed a structural arrangement of cellular structures and pseudocysts in good agreement. The TEM-study, however, gave a completely different picture of the internal structure of individual cells. We included case EH 255 in order to demonstrate that light microscopy showed a typical ACC while the TEM-study also disclosed many cells of a quite different type, full of atypical mitochondria (Fig 31). These mitochondria were found to remain after the pre-operative irradiation (4,000 rad) in the aspirated specimen. No tumor cells were found in the specimen taken for biopsy after the operation while simultaneous aspirates contained fairly numerous cells of the oncocytoid type. They had evidently been taken from different portions. The TEM-study also allowed a better differentiation between the individual cell-components of the tumour and of observation of nuclei and nucleoli as well as intercelluar contacts.

b) **Cytology and biopsy not in complete agreement**

This group includes three cases, namely:

Case No.	CYTOLOGY	HISTOPATHOLOGY	FROZEN SECTION
EH 230	Atypical PA alt ACC	ACC	PA
EH 237	Atypical PA alt ACC	ACC	PA alt Mucoep.ca.
EH 223	Highly suspected ACC	ACC	

The **cytologic smears** in the first two cases (EH 230 and EH 237) were rich in cells and displayed many adjacent or solid cell groups with mucoid red-violet strands, but in EH 237 no typical rounded mucoid globules could be seen. In EH 230 occasional, small globules were seen but not in sufficient numbers to confirm the diagnosis of ACC. During operation frozen sections were studied in these two cases and, as the table shows the diagnosis differed from the definite one made on paraffin sections.

The TEM-studies of the aspirate from EH 230 showed solid areas with L- and D-cells intermingled, but a clear dominance of D-cells, among which cells indicating squamous cell metaplasia were seen. There were comparatively few pseudocysts and

occasional true cysts. The TEM observation of this case with occurrence of PC and TC and the arrangement of L- and D-cells was in good agreement with an ACC but does not with our present knowledge exclude pleomorphic adenoma.

The TEM-study of EH 237 demonstrated a cell structure and arrangement which differed considerably from the other cases of ACC. The L-cells were atypical although the D-cells fitted the above description. The "L-cells" were very irregular, the nuclei displaying many indentations or clefts with pseudo-inclusions. The nucleoli were very prominent, the mitochondria in different stages of disintegration and there was a fairly rich endoplasmic reticulum, especially of the rough type. There were true cysts with microvilli but no pseudocysts. The squamous epithelium-like cells contained many tonofilaments. The picture obtained by examination of specimens from the operation showed agreement with the aspirate. In these sections desmosomes were also numerous between L-cells, while the aspirates demonstrated rather separated cells. In this case the TEM-study was not typical for ACC.

In the third case (EH223) the cytologic smear contained many dark and irregular cells. Red-violet mucoid strands pierced rather solid groups of epithelial cells. The smear contained a few PC-like globules. The histopathology in this case demonstrated a solid form of ACC and the few cribriform areas contained pseudocysts surrounded by several layers of epithelial elements. The TEM aspirate did not contain a very rich material. It contained L- and D-cells but was especially rich in intermediate cells. There was a pronounced hyalosclerosis. This is also in good agreement with the TEM-biopsy at operation which showed a very solid structure with a similar cell content and very many intermediate dark cells. There were few and small pseudocysts.

In conclusion this case represented a solid form and the paucity of globules in the cytologic smear made it difficult to confirm the diagnosis. Electron microscopy was in good agreement with ACC.

Thus the investigations show that these three cases, which were hard to evaluate in cytologic smears, were also difficult to classify histopathologically, nor did they reveal a uniform picture on TEM examination. The cases EH 230 and EH 223 were shown by histopathology to be a solid form of ACC with only a few cribriform structures — hence the divergent diagnosis of PA on frozen section examination of EH 230. The cytology was suspect, however, in that a few small "mucoid" globules were found. TEM examination showed typical PC structures in both cases. Case EH 237, which was atypical on both histopathological and cytological examination, displayed pronounced squamous epithelial metaplasia on TEM investigation, which may explain the inaccurate frozen section diagnosis of mucoepidermoid cancer. In general TEM examination of EH 237 revealed atypical ductal cells and no definite PC structures, so that the diagnosis ACC is doubtful.

c) **Cytology and PAD at variance**

Cytology: Highly suspect ACC (EH182). Atypical PA or ACC (EH 206)
PAD: Monomorphic adenoma (EH182 and 206)

Cytological smears for light microscopic examination were in both cases fairly uniform, being cell-rich specimens in which the cells lay for the most part in thick clusters. Here and there strands of reddish violet mucus pierced and enclosed the clusters. In places the mucus formed small distinct globules — occurring in great numbers in case EH 182.

Histopathological examination revealed a fairly similar cell picture in both cases, where the epithelial cells formed confluent, primarily solid but also small tubular structures, the latter being most prominent in case 182. Mucus staining elicited both positive PAS and Alcian green staining in both cases. In EH 182 no distinct tumour capsule was seen. In 206 there was a breach in the fibrous capsule over a small area.

The ultrastructural investigation of EH 182 demonstrated an abundance of, first and foremost, intermediate cells (both L_i and D_i), and cells showing squamous epithelial metaplasia. In addition many small PC-like structures were seen, usually not exceeding 1—2 cell diameters (15—30μ). The pseudocysts were surrounded by L-and D-cells, the L-cells had partly cleft nuclei and contained patches of granules. The L-cells were firmly linked with each other, and provided with numerous desmosoms formed solid clusters. The D-cells were more loosely scattered and only sparsely linked by desmosomes. The TEM examination of EH 206 showed predominantly L-cells, but squamous epithelial metaplasia was also seen. The L-cells had likewise partly cleft nuclei, containing granules here and there. The L-cells seem to be less strongly linked with each other than in EH 182, with large intercellular spaces. Moreover a moderate occurrence of smaller PC-like structures was seen as in EH 182. Various large "mucous lakes" were observed, which were also surrounded by a basement membrane-like structure.

Thus the investigation shows that it is possible to find the mucous globules observed in the cytological smears as PC-like structures in TEM. These PC structures, however, could not definitely be found in paraffin sections in the form of cribriform structures. Perhaps this finding in TEM may be manifestations of microadenoid-cystic cancer of a type similar to that described by Evans and Cruickshank (1970), where the tumour consisted of a mixture of a microadenoid cystic cancer and malignant basal cells, i.e. in this case it could be a monomorphic adenoma in the process of becoming a microadenoid cystic cancer.

A summary of the results from 13 cases with cytologically certain or suspect ACC

In the eight cases with **cytology typical of ACC** with findings of distinct mucous globules of varying sizes, these were found in the light microscopic thin sections in the form of the cribriform structure characteristic of ACC. Corresponding "pseudocysts" were also seen in TEM. The PC-bordering cells comprised L, D and intermediate cells.

In the five cases in which **ACC was cytologically highly suspected** the findings consisted of small, sparse, globular, distinct structures. Here the histopathological thin sections showed an overwhelmingly solid mode of growth. Nevertheless in all but two (EH 182 and EH 206) it was possible to discern small areas with cribriform growth, so that the ACC diagnosis was reached histopathologically. TEM proved the solid areas to be not constructed of a specific cell type — nor were the cells surrounding the PC structures homogeneous.

On TEM examination PC structures could be found in all cases except EH 237, thus even in EH 184 and EH 206, which were histopathologically described as monomorphic adenoma.

In case EH 237 the frozen section diagnosis also diverged from the final histopathological diagnosis, i.e. pleomorphic adenoma or mucoepidermoid cancer. TEM in this last case showed the L-cells to be atypical, in that they were abnormally cleft. No definite PC structures could be demonstrated. The future will probaly show whether the diagnosis monomorphic adenoma in cases EH 182 and EH 206 holds good, or whether ACC will be the definitive diagnosis, and also whether EH 237 really is an adenoid cystic cancer.

Consequently the examination of the 13 cases indicates that when a cytological diagnosis is considered certain with regard to ACC, histopathology and TEM will also show close agreement. On the other hand, when ACC is highly suspected from cytology this may imply the solid and therefore highly malignant form of ACC or it may be, as in this case, atypical monomorphic adenoma. Only continued follow-up can answer the question of whether findings of PC structures on TEM examination of these two cases of monomorphic adenoma signify that this is a histopathological wrong diagnosis. The possibility that a microadenoid-cystic cancer is here present has been mentioned. In some cases — apart from these 13 — where the cytological diagnosis was atypical pleomorphic adenoma these PC structures were likewise seen in TEM. Similarly ACC-like cytological pictures were observed on puncture of a resistence on the neck, where the histopathological thin section disclosed a nodular hidradenoma. Consequently further TEM studies on an increased material are called for in order to determine which structures in salivary gland tumours are pathognomonic for ACC. Before any decision can be reached on acceptance or rejection of the belief that discovery of PC structures on TEM examination is a characteristic finding for ACC in salivary glands. (Table IV).

CASE N:O	DIAGNOSIS		LIGHT MICROSCOPY FINDING		PAS	Alc. green	Congo red	TEM Observation (Aspirate)
	Fine-n. pct	Histopat.	Cytology	Histopathology				
EH 163	ACC	ACC	Mod MG— Dom L-cells	Abund So, Few Cr Mixed D & L-cells	—	+	—	Mod small & large PC. Mod TC. Dom L-cells
EH 164	ACC	ACC	Abund MG. Dom L-cells	Abund So. Mod Cr. Dom L-cells	—	(+)	+ +	Abund small and large PC. Mod TC. Dom L_i-cells
EH 167	ACC	ACC	Abund large MG.	Abund Cr.	—	+ + +	—	Mod PC. Dom L_i-cells
EH 214	ACC	ACC	Abund small and large MG. L & D-cells	Abund Cr.	(+)	+ + +	—	Abund large PC. L & D-cells. Few TC
EH 220	ACC	ACC	Few large MG. Dom L-cells	Abund Cr. Mod So Dom L-cells	(+)	+ + +	—	Few Pc. Dom L-cells
EH 225	ACC	ACC	Mod large MG. Undef cells	Multil Cr. L & D-cells	+	+ + +	—	Abund large PC. Few TC L_i & D_i-cells
EH 249	ACC	ACC	Abund small MG. Undef cells	Abund small Cr. Undef cells.	after X-ray (+)	— —	+ +	Abund PC. Sq-metaplasia
EH 255	ACC	ACC	Few large MG. Dom L-cells	Mod Cr. Undef cells	X-ray no staining			Mod large PC. Abund oncoc. L & D-cells
EH 223	Susp ACC	ACC	Few large MG. Undef cells.	Multil Cr. Abund So. Undef cells.	—	+ + +	—	Abund small PC. Mixed L_i & D_i.
EH 230	Atyp PA alt ACC	ACC	Few small MG. Undef cells.	Abund So. No typical Cr.	+	+ +	—	Few PC & TC. Cleft L. Sq-metaplasia
EH 237	Atyp PA alt ACC	ACC	No typical MG. Dom L-cells.	Abund So. No typical Cr.	+ + +	+ + +	—	No typical PC. Sq. Abund TC. Cleft L-cells
EH 182	Highly susp ACC	Atypical monom ad	Abund small MG. Dom L-cells	Abund So. Few Cr. Dom L-cells	+ +	+ + +		Abund small PC. Dom cleft L. Few TC
EH 206	Atyp PA alt ACC	Monom ad.	Few MG-like struct. Dom L-cells	Abund So. Few Cr. Dom L-cells	+ +	+	—	Few small PC. Dom cleft L. Few TC.

Table IV **Schematic presentation of the results from light and electron microscopic comparison of 13 cytological certain or suspect ACC.**

Abbreviations:
Abundant — Abund
Moderate — Mod
Few — Few
Mucoid globules — MG
Dominance — Dom
Cribriform — Cr
Solid — So
Squamous metaplasia — Sq
Multilayered — Multil
Undefined — Undef

d) **Semiquantitative examination of TEM sections from aspirates and corresponding surgery specimens**

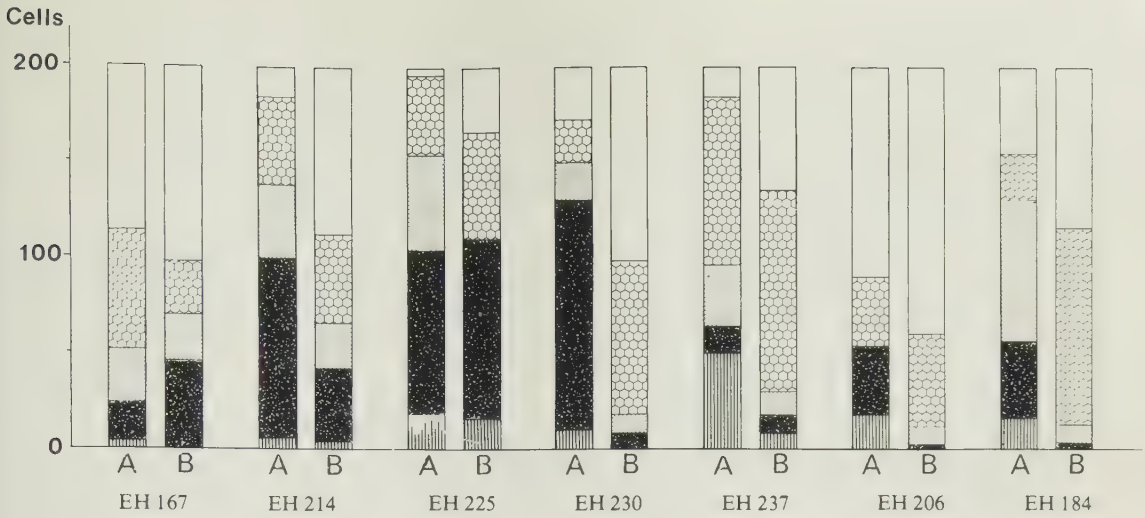
The following seven cases were examined:

Case No.	CYTOLOGY	HISTOPATHOLOGY
EH 167	ACC	ACC
EH 214	ACC	ACC
EH 225	ACC	ACC
EH 230	Atypical Pa alt ACC	ACC
EH 237	Atypical PA alt ACC	ACC
EH 182	Highly suspected ACC	Monomorphic adenoma
EH 206	Atypical Pa alt ACC	Monomorphic adenoma

From both the Epon-embedded aspirate and the surgery specimen from each case, a decision was first reached, by light microscopic examination of semi-thin sections, on the presence of tumour tissue. Two hundred neighbouring cells were then counted on TEM sections. Several sections were used if one did not suffice. These were taken at random. A histogram was then made for each preparation, Table V. We are aware that TEM examination only provides information on a very small part of the tumour, especially if it is of heterogeneous composition — this applies to TEM sections from both surgery specimens and aspirates. The purpose of the investigation, however, was not to carry out a morphometric examination, but rather a study to indicate whether the same kinds of cell were derived from surgery specimens (B) as from aspirates (A), and whether any cell type is lacking from both preparations.

The **results** of the investigation suggest that the aspirate (A) in six of the seven cases contains more D and D_i cells than were present in the corresponding surgery specimen. A study of the respective D- and L-cells in TEM reveals that the D-cells are often lying free and dissociated, surrounded by mucous material, being joined to each or to the L-cells only by a few bonds in the form of desmosomes. The L-cells, on the other hand, are often lying in large clusters, bound together by dense desmosomes. By reason of less pronounced cell cohesiveness, therefore, more D-than L-cells would probably be obtained by aspiration since the former presumably detach more easily. The same would appear to be true of the squamous epithelia which are likewise found in greater numbers in the A preparations. The presence of PC structures — although small and of rare occurrence — in the aspirates of all cases of ACC examined is probably due to the fact that mucous globules are very easily aspirated, being the first structures to detach.

The histograms show that fairly large numbers of intermediate cells appear in both A and B preparations. The findings are in accord with the opinion of Kleinsasser et al. (Zur Histiogenese der Cylindrome 1969): "Im übrigen sei bemerkt, dass allen lichtmikroskopischen, histochemischen und elektronoptischen Befunden, die Mehr-



L — cells (lights cells)
D — cells (dark cells)
L_i — cells (light intermediate cells)
D_i — cells (dark intermediate cells)
S_q — cells (Metaplastic squamous epithelium)
A — Aspirate
B — Biopsy

Table V. Histogram demonstrating how different cell types are represented in aspirates (A) and surgical biopsies (B). As can be seen, both A and B contain corresponding cell groups but the aspirates have a clear tendency to contain more D-cells than the biopsies.

zahl der Zellen in einem Cylindrom (ACC) sich offenbar in einem primitiven Differenzierungs-stadium befinden. Nur zu einem relativen kleinen Teil sind die Zellen schon so weit entwickelt, das sie eindeutig als drüsig oder myoepithelial identifizierbar sind. Seiner Differenzierung nach entspricht das Cylindrom demnach einer primitiven fetalen Adenomere, in der eben die Entwicklung in drüsige und myoepitheliale Zellen einsetzt".

If we venture to draw any conclusion from the distribution of the cells, the L-cells seem to dominate the surgery specimens, while no definite tendency is discerned in aspirated preparations. Thus the surgery specimens show the same trend as was demonstrated by Chisholm et al. (1975), who described a clear predominance of duct-type cells (corresponding to the L-cells in this investigation), which amounted to 75 vol%. The myoepithelial cells (the D-cells) amounted to only 3 vol%. This is in contrast to the investigations of Bauer & Fox (1945), who considered that the myoepithelial cells dominated the tumour. Various other authors are of the same opinion as Bauer (Thackray & Lucas 1960, Friborsky 1963, Hübner et al. 1969), although only Chisholm et al. have carried out quantitative studies.

Thus no clear dominance of any cell type is seen in our tumours, at least not in the aspirate. Nevertheless all tumours consist in principle of the same kind of cells, i.e. L, L_i, D_i and D together with Sq cells, and each individual tumour has its special pattern. Similarly varying amounts of mucous substance are seen (see Table IV in previous chapter) with different staining properties, presumably indicating different chemical composition.

e) **Findings in two irradiated cases of ACC,** EH 249 and EH 255

In these two cases light microscopic cytology revealed typical ACC with epithelial cells lying both isolated and in solid clusters, where the cell population in the first case (EH 249) appeared to be dominated by comparatively large, round to oval cells with varying chromatic density. A moderate number of much smaller cells with hyperchromatic nuclei and irregular membranes were also found. The cytoplasm in the larger cells was indistinct and poorly defined. In the second case (EH 255) the picture was dominated by comparatively large, light, round or oval cells, which usually displayed a fairly large nucleolus in the nucleus. The cytoplasm was well defined and of a beautiful blue colour, in contrast to the lighter, almost indiscernible cytoplasm in the first case. Both specimens presented an abundance of red-violet mucus, which formed distinct, small (EH 249) and large (EH 255) globules of the type seen in the other cases of ACC confirmed by PAD. Moreover the mucus here and there formed structures resembling ductal casts. Semi-thin sections of the Epon- embedded aspirates were obtained in sufficient quantity from EH 249 before irradiation and after irradiation with 2.000 and 4.000 rad (Fig. 30A). From EH 255 material was taken before and after irradiation with 1.000, 2.000, and 4.000 rad (Fig. 31A).

TEM examination of the material before irradiation showed typical ACC structures with pseudocysts (Fig. 30B and 31B). After irradiation with 1.000 and 2.000 rad a certain general dispersion appeared, most pronounced in EH 249. The tumour cells were dispersed, surrounded by an amorphous, unstructured material, probably corresponding to the reddish violet mucus seen in light microscopic smears from the same cases.

After irradiation with 4.000 rad the dispersion in EH 249 increased even further, as shown by Fig. 30 C and D. On TEM examination PC structures could still be observed, but the membrane began to lose its sharpness and the cytoplasm of the cells showed signs of degeneration. The nuclei were frequently dispersed and indistinct.

Although the light microscopy showed a typical ACC-picture with pseudocysts in case EH 255, the TEM study of material obtained before irradiation demonstrated a

dominance of rather large cells resembling L-cells, almost all of which contained considerable amounts of densely packed mitochondria of a type often seen in oncocytoid tumours. We never before observed this agglomeration of mitochondria in cases of ACC. The cristae of the mitochondria do, however, differ slightly from what we generally find in oncocytomas, and are partly of the tubular form. (Fig. 31 C and D) PC:s being numerous. The finding of these agglomerations of mitochondria with atypical cristae is not from our point of view in full agreement with the light microscopic diagnosis and a metastatic process must be considered.

Even after irradiation with 4.000 rad many cells were still present and the mitochondria remained little changed. Moreover PC:s were present. In this case biopsy material did not reveal any remaining tumour cells, evidently because different regions were studied.

These two cases are of interest from different points of view. Under light microscopy they are very similar and the diagnosis was ACC. TEM demonstrated considerable differences, however, and indicated in one case the possibility of some other kind of tumour. Furthermore the repeated aspirates from the tumours during treatment gave information concerning the effect of the irradiation. These preliminary observations thus stimulated us to further studies, now in progress, of the effect of irradiation.

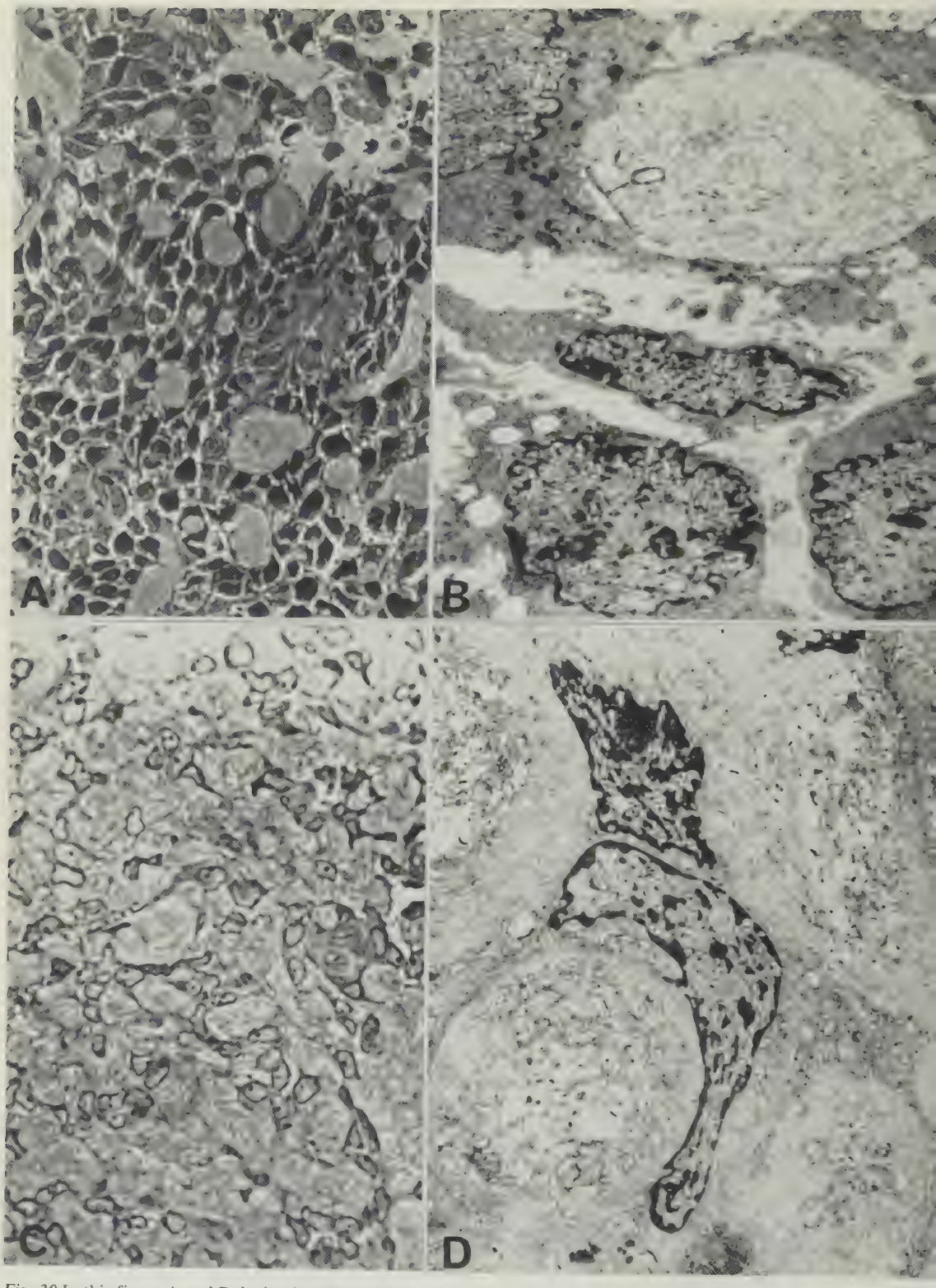


Fig. 30 In this figure A and B depict the structure of an ACC before irradiation. A and C are semi-thin sections and B and D TEM. It can be seen how the normal texture of A is modified in C with reduction of cells per square surface and increase of mucus. In B it can be seen how the PC-configuration is distinct while D shows indistinct borders and certain modification of nuclear structure. (A 600×, B 8 000×, C 600×, D 8 000×). C and D after irradiation 4 000 rad.

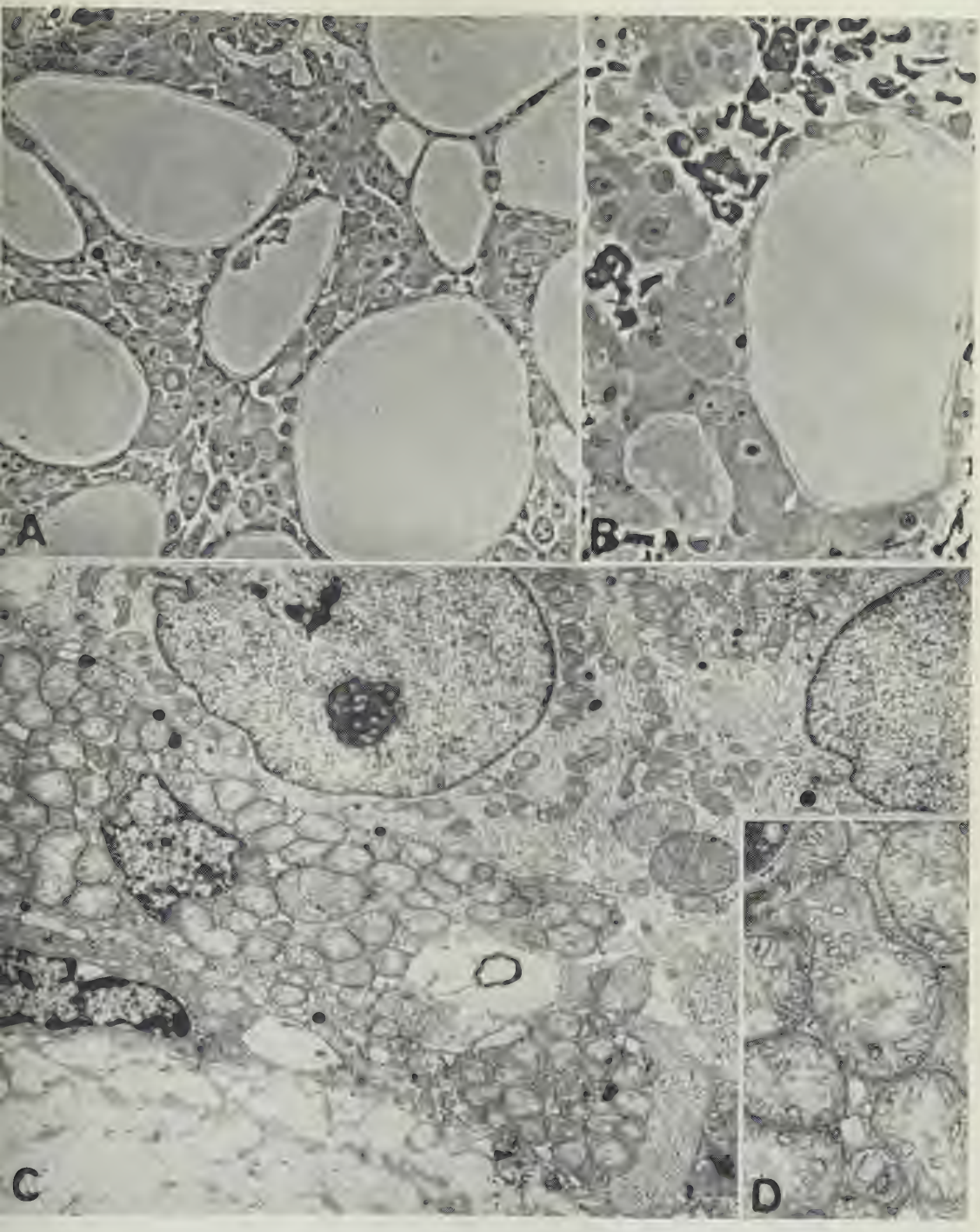


Fig. 31 Adenoid cystic cancer (Case EH 255). In this controversial case the micrographs A-D demonstrate the morphology of the tumour cells. The cytoplasm contains large number of atypical mitochondria with tubular cristae. The micrographs A and C are taken from an aspirate before irradiation, B is after 4000 rad. It can be seen that the irradiation causes indistinctness of cytoplasm and cell boeders. (A 200×, B 300×, C 10000×, D 22100×).

Part IV GENERAL DISCUSSION

Fine needle biopsy has become an important tool widely used in Sweden and many other countries for the diagnosis of tumours. The technique is based in principle upon aspiration of tumour material by mean of a specially designed syringe and a thin needle. The aspirated material is spread on an object glass, stained and studied with the help of microscopy. Such a study can provide vital information about the type of tumour present. Fine needle biopsy has proved to be of great value particularly in tumours not easily accessible for conventional biopsy. Indeed in salivary gland tumours, much discussed in the present publication, fine needle aspiration has been our standard pre-operative form of biopsy for more than a decade. A series of publications have already demonstrated the great value and slight risk inherent in this method.

In clinical practice postoperative studies of the histopathology of extirpated tumours have been constantly used and trusted in the final diagnosis of the tumours under discussion. Pre- and postoperative diagnosis have been clearly shown to yield results in very close agreement.

When publications repeatedly appeared, reporting on transmission electron microscopy (TEM) studies of the material obtained by conventional biopsy it seemed natural to investigate whether TEM could also be used in the examination of the material derived from fine needle biopsy. In 1973 Hagelqvist and Engström published material clearly indicating that TEM studies of aspirated material could also provide further information on tumours. The preliminary paper pointed out that several parameters involved in the handling of the material were of importance for the quality of the results. During the years following this early paper the material here published was obtained and several of these parameters examined. Some of the problems inherent in such a study are described in the Introduction. Various major issues concern the quality and quantity of the material obtained, together with the possibility that TEM can provide important information in addition to that derived from light microscopy. We are convinced that this is the case.

It must be pointed out, however, that many factors which have become extremely interesting for the structural analysis of tumour cells, such as mitochondria, endoplasmic reticulum, cell borders and cytoplasmic inclusions of various kinds are difficult to study in detail with light microscopy, while TEM with its higher resolution immediately distinguishes between e.g. cytoplasmic secretory granules and mitochondria. Transmission electron microscopy also allows detailed observation of particles such as viruses, or study of cell borders and cell junctions. Every study demanding high resolution immediately raises the question of how the specimens should be treated to give good quality.

Another factor may be very inportant in clinical work, namely the need to produce good quality within a short time. As described in the previous chapters we approached several of these problems in animal experiments and in studies on human material. Diverse other questions which we consider relevant to our work are described in the Introduction.

In **animal experiments** we first made aspirations from liver and salivary glands, both of which have a well known structure. It became very clear that distinct structural details could be observed, and the method gave a TEM picture in good agreement with the standard picture of both liver and salivary gland cells. Later studies of human material confirmed our animal results. The animal experiments were continued in a study of problems related to the quantity of material obtained by fine needle aspiration. The background of this study was obvious. In 20 years of work on fine needle aspiration cytology we have learnt that certain tumours may yield large numbers of cells while other tumours produce but few. We therefore studied material from liver, which we regarded as an "easy" material, and compared the number of cells with material from kidneys which we considered "difficult". Liver obviously gave aspirates much richer in cell content. We therefore started to investigate different kinds of needles to be used in specimen collection from different kinds of tumours. We feel that special grinding of the needle opening allows us to obtain a richer material than with ordinary needles, and believe that different types of needle must be used for the best possible results. We concentrated on liver and kidney material and a statistical analysis of our results indicates that the amount obtained from aspiration with specially constructed needles can be richer than material collected by conventional needles of the same diameter. Nevertheless other factors such as the risk of haemorrhage and trauma must be taken into account when new needles are developed. We also compared e.g. needle length and needle diameter and discovered that an ordinary 0.8 mm diameter needle gives more material than an 0.7 mm specially ground needle. On the other hand, it seems to cause more bleeding so that we prefer the thinner needle.

We also studied the importance of the length and found that a longer needle does not necessarily give more material than a shorter. Our studies merely indicate the importance of the design of the needle. We are continuously studying this factor which we believe to be important for certain types of tumour. In the present investigation we based our observations on a comparison of dry weights of material from different kinds of needles. It is not easy clearly to define the weight of the material, since contamination with blood and other fluids may confuse the results. To reduce the risk of excess blood in those cases where sharp or coarse needles were used, we carried out our measurements on material freshly removed from animals. We are well aware that this question can be much more carefully analysed but we used our results as a pilot study.

Using animal and human material we have examined fundamental problems encountered in the preparation of aspirates for TEM-studies. The factors concerned handling of the specimens, prevention of contamination and delay, compared with

other methods. We used two different methods for fixation and embedding, a standard technique for ordinary cases and a rapid method for those cases in which a rapid diagnosis is important. Both methods are based upon techniques commonly used in TEM-studies but adapted to our needs. To prevent contamination and loss of material all handling of the specimen from fixation to embedding in Epon, takes place in a single jar — a thin glass tube. The time span from aspiration to electron-microscopic observation can be reduced to 5—6 hours with this rapid technique. We regard this time as satisfactory in most cases. With this technique we have for instance been able to show within five hours after aspiration the presence of viruses.

Another factor inherent in the technique was the great importance of selecting appropriate areas for TEM-studies by a scrutiny of large semi-thin (1—2μ) sections. When these were stained we found that the large semi-thin sections often provided information on the arrangement of the cells, the cell nuclei etc. These sections thus had a twofold value, both for selection of a special specimen area and for information on the structure and arrangement of the tumour cells.

The human material used in this publication comes mainly from head and neck tumours, especially salivary gland tumours. The material of about 300 tumours and inflammatory processes was studied with the help of different techniques. As already discussed, we used both conventional techniques for cytological smears and biopsy and TEM. In the present study less attention was paid to the differential diagnoses of the tumours or their exact classification than to the possibilities obtaining information on the tumour cells exceeding that procurable by light microscopy. It is our firm belief that the intracytoplasmatic structure of tumour cells, the size and shape of their nuclei and their cell borders are of importance for our understanding of the tumours and presumably for their classification in the future.

In the differentiation between different intracytoplasmic structures such as secretory granules and mitochondria, light microscopy may often present certain difficulties while TEM affords immediate separation. TEM may also provide important information on the individual form or structure of, for instance, mitochondria and on several occasions we were given essential assistance in the diagnosis by the structure of the mitochondria. One of these cases was especially interesting. A woman with tumour in her left parotid gland was operated. The diagnosis was acinic cell cancer. After some time she rather suddenly manifested tumours in the parotid gland on the other side and operation once again revealed acinic cell cancer. The specimens were studied by several pathologists and the diagnosis was unanimous. Because of the findings at TEM cytology which showed a structure differing from that of an acinic cell cancer, which normally has large numbers of secretory granules, the diagnosis was questioned. The aspirated material showed an uncommon form of mitochondria (Fig. 21 B-D) which greatly resembled mitochondria found by Seljelid & Ericsson (1965) in renal carcinomas. An investigation showed bilateral renal tumours the diagnosis being confirmed by angiography and fine needle biopsy.

Other cytoplasmic structures may be much easier to discern by TEM than by light microscopy. This was the case in various amelanotic melanomas where TEM disclosed characteristic premelanosomes, not observed by light microscopy. As our knowledge of the structures of premelanosomes is not yet sufficient, difficulties may arise even with TEM. In a case of undifferentiated cancer in the parotid region with rapid invasion of cranial nerves and meninges the light microscopic aspiration cytology suggested malignant melanoma as a possibility, but the pathologists characterized the tumour as undifferentiated carcinoma. As can be seen in Fig. 26 E the tumour contains crystalloid structures with a certain resemblance to premelanosomes. The course of the patient's disease is in close agreement with either of the two possible diagnoses.

The present TEM-study has clearly demonstrated the possibility of obtaining cell complexes containing many well fixed tumour cells in relation to each other and of studying the interior of the cells at high resolution. Many factors are of particular interest. Thus the cell surface and the cell borders or cell junctions can be well observed and depicted.

During the fairly short period in which the fine-needle aspirates have been used the opposition to the method has been based on the assumption that the small amount of material obtained by means of aspiration would not be representative of the tumour as a whole. This is contradicted, however, by the good results, in term of diagnosis, reached by many investigators. The increased number of laboratories using the techniqe have demonstrated the close agreement between pre-operative cytology and histopathology on ordinary biopsy material (Eneroth *et al.* 1967 for salivary gland tumours, Lundqvist 1971 for liver biopsies). Since the TEM- investigation following fine-needle aspiration is based upon the same kind of material the material could be expected to have a corresponding representativity. It could of course be said that the TEM-specimen is only one small portion of the aspirate but this is also true of a biopsy specimen used for TEM. The semi-thin sections are therefore of great interest, as they show many more cells and permit a localization to interesting areas. The semiquantitative study carried out on tumours of ACC-type, described in Part III, clearly demonstrates that all kinds of cells found at histopathology are also discovered at TEM-cytology. This study also indicates that the aspirate contains fewer cells with many desmosomes (L-cells) and more with few junctions (D-cells) than the biopsy specimen. This could of course be expected as highly attached cells will have a lower tendency to contribute to the aspirate. It is possible that a small biopsy can be less representative than an aspirate which includes cell groups from several parts of, for instance, a lymph node.

In ACC it is typical that mucous globules, which are very important for a light microscopic diagnosis, usually are very well represented as pseudocysts on TEM observation.

Several authors have indicated that the underpressure used at aspiration could cause cell damage. This is refused by viability- studies made by Criborn et al. (1964), and studies on imprints versus aspirates performed by Stenqvist et al. (1977).

The present study has also shown that the structure of the cells and cell organelles is usually very well preserved. Thus it seems that the aspirate can be regarded as representative of the cell population in a tumour, even if the proportions of different cells differ from those obtained at biopsy. It is also evident that we usually obtain very many cells in the aspirate, and that the morphology of the individual cells seems to be well preserved.

In this study our main purpose was to develop and study a new technique. The present publication demonstrates some of the possibilities but further work is needed to elucidate its whole value. We regarded it highly promising that the technique allows the study of the interior of single cells or large cell groups. The diagnostic value of the method is already evident in several cases, where electron microscopy of cytological specimens gave reason to doubt the diagnosis from biopsy, and where further studies have verified the cytological diagnosis.

Part V SUMMARY

Twohundred and ninety cases of "tumours" from the head and neck region were studied in the present investigation. A large proportion (160) consists of salivary gland tumours, but the material also contains various cases with infections, sialosis, mycosis, metastatic nodes, a myeloma, one eosinophilic granuloma, three malignant melanomas etc. In these cases cytology and light microscopy were combined with transmission electron (TEM) according to methods described.

A large proportion of the thesis is devoted to a description of the techniques used and their possibilities. It is stressed that these techniques can be improved.

Various questions were posed in the introduction and we believe that the results permit us to state:

that TEM-studies on material obtained by fine needle aspiration can be carried out with good results as judged in qualitative terms,

that the TEM aspirates generally contain sufficient material for a representative evaluation of the different cellular components of a tumour,

that the TEM micrographs provide important information on the individual cells and their organelles, which is often so valuable as to give essential clues for clinical diagnosis,

that in certain cases TEM-studies can easily distinguish types of tumours which may be difficult to differentiate in light microscopy. A typical example is acinic cell cancer versus oncocytoma,

that TEM-observations of a specimen can if necessary be carried out, with a special technique, within 5—6 hours after aspiration,

that since aspiration of specimens can be repeated several times with little risk or discomfort for the patient, TEM-studies can be used to follow and evaluate the results of a treatment using irradiation or cytotoxic drugs,

that TEM-studies on aspirates should be further used to obtain a better opinion concerning the value of the method from a diagnostic point of view. We believe that such studies could make a major contribution to our knowledge of various types of tumours and of their cellular components. The extent to which TEM-studies will eventually contribute to a better classification of tumour groups is still difficult to define but in our studies we have found TEM made on aspirates to be an important adjunct to other methods used in the pre- operative classification of tumours.

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